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MICROVESICLES IN PLATELET CONCENTRATES FOR TRANSFUSION

A thesis submitted to the Open University by

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for the degree of Doctor of Philosophy

Sponsoring Establishment

Addenbrooke 's NHS Trust

Collaborating Establishment

National Blood Service - London and South East Zone

20 March 2000


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DECLARATION

The work described in this thesis was carried out under the supervision of Dr Jerard Seghatchian and Dr Lorna M Williamson at the National Blood Service - London and South East Zone between October 1997 and March 2000. It is entirely the result of my own work. This thesis has not been submitted, either in whole or in part, for a degree, diploma or other qualification at any other university.

A handwritten signature in cursive script, reading 'P. Krailadsiri'.

Pranee Krailadsiri, MD

20 March 2000

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LIST OF PUBLICATIONS

1. Microvesicles in blood components: Laboratory and clinical aspects.
Krailadsiri P, Seghatchian J, Bode AP. Clin Appl Thromb/Hemosta 1997;3:86-95.
2. The platelet storage lesion.
Seghatchian J, Krailadsiri P. Transfus Med Rev 1997;11:130-144.
3. Leucocyte filtration of platelet concentrates reduces red cell-derived but not platelet-derived microvesicles.
Krailadsiri P, Seghatchian J, Williamson LM. Transfusion 1999;33:15S.
4. Are all leucocyte depleted platelet concentrates equivalent?
Krailadsiri P, Seghatchian J. Vox Sang 2000, in press.
5. Platelet storage lesion of leucocyte depleted buffy coat-derived platelet concentrates prepared by three types of in-process filters/storage bags.
Krailadsiri P, Seghatchian J, Williamson LM. (submitted for publication)

ABBREVIATIONS

Abbreviations not define in the text

BSA	bovine serum albumin
Ca	calcium
CaCl ₂	calcium chloride
EDTA	ethylenediamine tetraacetic acid
kD	kilo Dalton
OD	optical density
rpm	revolutions per minute
Tris	tris-hydroxymethyl-aminomethane

ABSTRACT

The key objective of this study was to examine whether leucocyte depletion generated or removed platelet-derived microvesicles in platelet concentrates for transfusion. Three in-process leucocyte removal filters for pooled buffy coat derived platelet concentrates, i.e. negative charged polyester, positively charged polyester, and non-charged polyurethane, were compared. The effects of three major leucocyte depletion technologies currently in use in the UK, i.e. Cobe LRS and Haemonetics MCS+ LD apheresis, and filtration of pooled buffy coat derive platelets, on platelet microvesiculation were also examined. Furthermore, the effects of various leucocyte filters and leucocyte depletion technologies on platelet activation and the activation of coagulation/complement systems were investigated. The procoagulant and anticoagulant properties of microvesicles isolated from platelet concentrates were explored.

Leucocyte filtration of pooled buffy coat derived platelet concentrates by all three filters did not have a net effect on the level of microvesicles. All three leucocyte depletion technologies gave similar values of microvesicles on day 1, but on day 5 MCS+ LD apheresis showed the lowest value, whilst Cobe LRS apheresis and buffy coat methods were equivalent.

Among the three filters, the negatively charged filter activated the coagulation system as measured by kallikrein-like and thrombin-like activities, but removed some activated complement C3a, whereas the positively charged filter generated C3a. Among the three leucocyte depletion technologies, platelets prepared by Cobe LRS showed the lowest degree of activation of the coagulation system. However, both Cobe LRS and MCS apheresis showed higher levels of C3a than filtered buffy coat derived platelets.

The microvesicles isolated from day 1 platelet concentrates could act as a

catalytic surface for both the coagulant and anticoagulant reactions as measured by the formation of prothrombinase complex and the inactivation of FVa by activated protein C. The microvesicles isolated from day 5 platelets showed an increased procoagulant activity, whereas the anticoagulant activity substantially diminished.

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GENERAL INTRODUCTION

1.1 PHYSIOLOGY OF HAEMOSTASIS

Normal haemostasis embodies three interrelated compartments: vasculature, platelets and coagulation. The physiology of each compartment is briefly discussed below. Detailed description of all the changes at the molecular level are beyond the scope of this introduction.

1.1.1 Vasculature

The wall of a blood vessel is comprised of three layers: intima, media and adventitia. The intima consists of a monolayer of endothelial cells and an internal elastic membrane. The media consists of smooth muscle cells. The thickness of the media varies depending on the type, i.e. arterial or venous, and size of the blood vessel. The adventitia is composed of an external elastic membrane and supportive connective tissue.

Three important properties are responsible for the pathophysiological role of vasculature: permeability, fragility and vasoconstriction. Increased vascular permeability results in blood leaving the blood vessel and manifests as petechiae, purpura or ecchymoses in severe cases. Increased fragility can lead to the rupture of the blood vessel also resulting in petechiae, purpura, ecchymoses or deep-tissue haemorrhage. Vasoconstriction is controlled by local factors e.g. temperature; pH; pCO₂; sympathetic nervous system; and humoral factors e.g. epinephrine, norepinephrine, adenosine diphosphate (ADP), kinins, thromboxanes, fibrin(ogen)-degradation products (FDP).

When injury to blood vessels occurs, a reflex vasoconstriction produces marked reduction or shunting of blood flow. The injury to vascular endothelium also results in

the exposure of subendothelial collagen and basement membrane, platelets are immediately recruited to fill the endothelial gap and form a primary haemostatic plug. Concurrently, cells from the media layer differentiate and migrate through the internal elastic membrane and eventually differentiate into new endothelial cells to complete a reparative process. (Bick 1993, Jaffe 1995)

Subendothelial collagen and basement membrane as well as the exposed tissue factor can activate platelets and coagulation system as described below.

1.1.2 Platelets

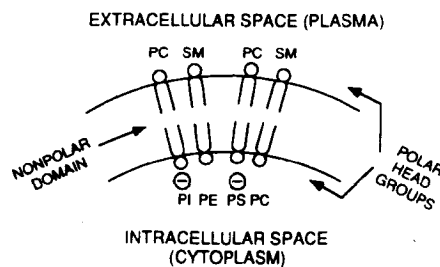
i. Morphology and biochemistry

The platelets in peripheral blood are heterogeneous in size, density and morphology depending on the anticoagulant, temperature and method by which they are examined. The resting platelet is disc-shaped. Using a rheo-optical method, in which platelets are measured while rotating freely in suspension, the dimensions of resting platelets are $3.6 \pm 0.7 \mu\text{m}$ in diameter and $0.9 \pm 0.2 \mu\text{m}$ in thickness, with a mean platelet volume range from 4 to 8 fL.

The platelet membrane consists of a bilayer of phospholipids. The distribution of phospholipids in the resting platelet is asymmetrical as shown by a simplified diagram in Figure 1.1. The outer leaflet consists mainly of phosphatidyl choline and sphingomyelin whereas the inner leaflet contains most of the negatively charged phospholipids, in particular phosphatidyl serine and phosphatidyl ethanolamine (Perret 1979). The mechanisms for the maintenance of an asymmetric distribution of phospholipids in platelet membranes are thought to be the selective affinity of cytoskeletal proteins for aminophospholipids (phosphatidyl serine and phosphatidyl ethanolamine) and/or the existence of an aminophospholipid-specific translocase activity which is ATP dependent and modulated by Ca^{2+} (Schrott 1991). The latter actively transports aminophospholipids from the outer to the inner membrane. During platelet activation the aminophospholipids,

particularly phosphatidyl serine, become exposed on the outer leaflet of the platelet membrane and act as a procogulant catalytic surface as described later in this introduction.

Figure 1.1 Asymmetric distribution of bilayer phospholipids of a resting platelet.



PC phosphatidyl choline, SM sphingomyelin, PI phosphatidyl inositol, PE phosphatidyl ethanolamine, PS phosphatidyl serine

The lipid bilayer, in particular the inner leaflet, also serves as a reservoir for two important substrates, phosphatidyl ethanolamine and phosphatidyl inositol. Phosphatidyl ethanolamine is the substrate for arachidonic acid used in prostaglandin synthesis (Schick 1981), and phosphatidyl inositol is a substrate for inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol, the important intracellular second messengers (Siess 1986).

Extending from the platelet surface is an extramembranous glycocalyx layer, which is composed of absorbed plasma proteins, membrane glycoproteins, mucopolysaccharides and sialic acids. The surface of platelet has a number of indentations which are the opening of the open canalicular system (surface-connecting tubular system). This tubular system is lined with the same membrane as the external surface of platelet membrane. During the release reaction, platelet granules fuse with

either the plasma membrane or the open canalicular system, and the substances in platelet organelles are then extruded to the exterior.

Encircling the periphery of the platelet are microtubules, microfilaments, and actomyosin (thrombosthenin) which act as a cytoskeleton for the platelet plasma membrane maintaining resting platelets in their characteristic discoid shape (White 1993). The dense tubular system is also seen at the periphery of platelets. The dense tubular system contains primarily adenine nucleotides and calcium. It can sequester and release ionised calcium when platelets are activated. It may also be a major site of prostaglandin synthesis.

A schematic representative of a resting platelet as seen by electron microscopy is shown in Figure 1.2.

The cytoplasm of the platelet contains several organelles: dense bodies, α -granules, mitochondria, lysosomes and glycogen granules. The major contents of dense bodies, α -granules and lysosomes are shown in Table 1.1.

Figure 1.2 **A schematic representation of a resting platelet as seen by electron microscopy.**

Left-equatorial plane, right-cross-section (From Isenberg WM, Baintain DF. In: Hoffman R, Benz EJ, Shattill SJ, Furri B, Cohen HJ, Sulberstein LE, eds. Hematology: Basic principles and practice, p1516-1524.)

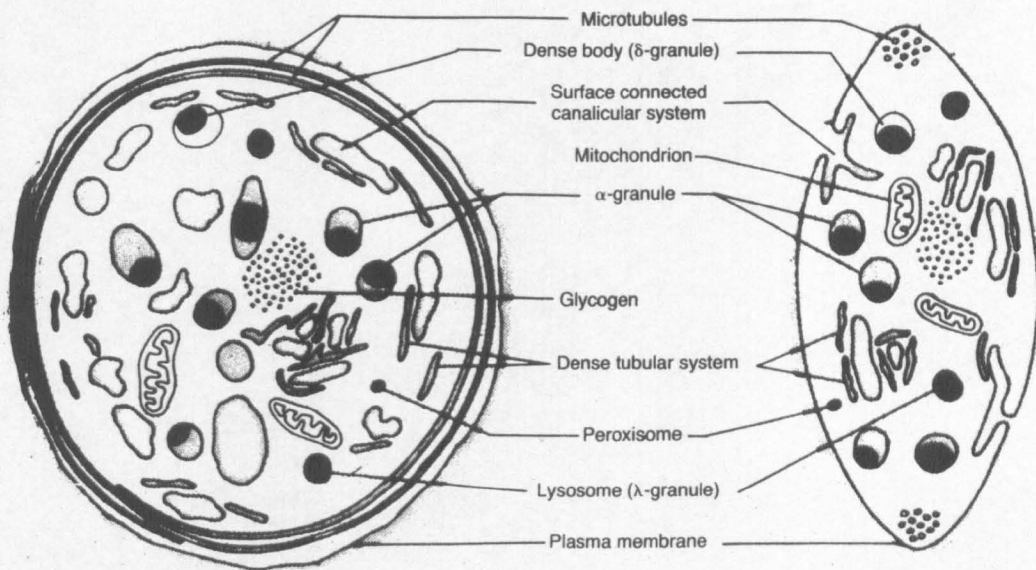


Table 1.1 **Major contents of dense bodies, alpha granules and lysosome.**

Organelles	Contents
Dense bodies	Nonmetabolic adenine nucleotides, serotonin, catecholamines, secretable Ca^{2+}
Alpha granules	Platelet specific proteins: PF4, β TG, PDGF, thrombospondin Nonspecific (plasma) proteins: Fibrinogen, fibronectin, albumin, factor V, plasminogen, HMWK, vWF, α 2-antiplasmin, α 1-antitrypsin, α 2-macroglobulin
Lysosomes	Acid hydrolases

PF4 - platelet factor 4, β TG - β thromboglobulin, PDGF - platelet derived growth factor,
HMWK - high molecular weight kininogen, vWF - von Willebrand Factor

ii. Platelet proteins

Platelets contain several proteins, some of which are common to plasma, but some are unique to platelets and present in only trace amounts in plasma. Some platelet proteins which have major roles in haemostasis are described below.

a. Platelet factors

So far platelet factors 1 to 7 have been identified. The most important are platelet factor 3 (PF3) and platelet factor 4 (PF4). PF3 (platelet thromboplastin) is required in at least two steps in blood coagulation: the activation of factor X by factor IXa and factor VIIIa, and the formation of prothrombinase by the interaction between factor Xa and factor Va (Rosing 1988, Schrott 1991). As PF3 is made available on platelet membranes when platelets are activated, it has been suggested that it is associated with the exposed aminophospholipids.

PF4 (antiheparin factor) binds to heparin with high affinity and neutralises heparin's anticoagulant activity.

b. β -thromboglobulin

β TG also binds to heparin but with lower affinity than PF4, thus β TG neutralises heparin less well.

c. Thrombospondin

Thrombospondin is an adhesive glycoprotein present almost exclusively inside platelets. It binds to several receptors on platelet membrane including GPIIb/IIIa, proteoglycan, GPIV (CD36) and fibrinogen. Thrombospondin appears to stabilise formed platelet aggregates and may also modulate fibrinolysis.

d. Platelet actomyosin (thrombosthenin)

This protein functions in most aspects similarly to smooth muscle. An increase in cytoplasmic Ca^{2+} leads to the activation of myosin and the conversion of monomeric actin to filamentous actin. Polymerisation of filamentous actin and myosin provides the

mechanical force for various phenomena involved in platelet activation i.e. shape change and release reaction.

e. Platelet glycoproteins

Platelet glycoproteins are important structural components of the platelet membrane that interact with the vasculature, other platelets and plasma proteins. The major glycoproteins on platelet surface membrane, lysosomal, alpha, and dense granule membranes are summarised in Table 1.2 and described below.

GPIa/IIa (CD49b/CD29)

Platelet adhesion to collagen mediated by GPIa/IIa requires magnesium and is inhibited by calcium. The interaction is not dependent on vWF.

GPIb/GPIX (CD42)

GPIb is composed of GPIb α (CD42b) bound to GPIb β (CD42c) with a disulphide bond. GPIb extends much further out from the platelet surface than does GPIIb/IIIa, which may account for its major role in platelet adhesion. This long extension may also make it susceptible to conformational changes induced by shear forces. The extracellular region of GPIb α can be cleaved by several proteases including platelet calpains, yielding a soluble fragment called glycocalicin that circulates in normal plasma at 1 to 3 $\mu\text{g/ml}$. The cytoplasmic domain of GPIb α connects to actin-binding protein, so alterations in the platelet cytoskeleton can affect GPIb functional activity.

GPIb appears on the surface of platelets in a one-to-one complex with GPIX. The function of GPIX is unknown but it is probably required for efficient surface expression of GPIb.

GPIb mediates platelet interaction with vWF via the region which does not contain Arg-Gly-Asp (RGD) and unlike GPIIb/IIIa does not require platelet activation. Plasma vWF will not bind to GPIb unless the antibiotic ristocetin or the snake venom

botrocetin is added. However, when vWF is immobilised on a surface, it may undergo a conformation change, allowing direct interaction between GPIb and vWF, even in the absence of ristocetin or botrocetin.

GPIc/IIa (CD49e/CD29)

GPIc/IIa mediates the adhesion of resting platelets to fibronectin. The biological role of these glycoproteins is not clear. It may involve in the binding of megakaryocytes to bone marrow matrix.

GPIIb/IIIa (CD41/CD61)

There are 40,000 to 80,000 GPIIb/IIIa molecules on the surface of a resting platelet. Another 20,000 to 40,000 molecules are present inside platelets, primarily in α -granule membranes and the membranes lining the open canalicular system. These can join the plasma membrane upon platelet activation and release reaction.

On resting platelets, GPIIb/IIIa has a low affinity binding for soluble fibrinogen. When platelets are activated with various agonists, changes in GPIIb/IIIa occur, leading to strong binding to fibrinogen. Binding of fibrinogen to platelet GPIIb/IIIa leads to platelet aggregation via cross-linking of GPIIb/IIIa on two different platelets by fibrinogen. In addition to fibrinogen, upon platelet activation, GPIIb/IIIa can bind to several other adhesive proteins containing RGD sequences. These include vWF, fibronectin and thrombospondin.

GPIV (CD36)

GPIV has been proposed as a platelet receptor for thrombospondin and collagen but its functional significance remains unclear as no clinical symptoms have observed in individuals who lack GPIV.

GPV

GPV appears to form a non-covalent complex with GPIb/IX. However, since the number of GPV molecules is approximately 50 percent of the number of GPIb/IX

molecules, it has been suggested that the complex consists of two GPIb molecules, two GPIX molecules, and one GPV molecule. GPV possibly involves in the expression of GPIb/IX.

GPVI

The binding of GPVI to collagen occurs as a "two-site, two-step" interaction. The binding of GPIa/IIa to collagen allows the binding of GPVI. The interaction between GPVI and collagen induces platelet activation via the phospholipase C pathway leading to the generation of second messengers in platelets (Pasquet 1999, Watson 1999).

P-selectin (GMP140, PADGEM, CD62P)

There are 2,500 molecules of P-selectin on the surface of a resting platelet and 20,000 molecules in the membrane of α granules which fuses with the plasma membrane when platelets are activated. The expression of P-selectin on the platelet membrane has, therefore, been used as an indicator of platelet activation (McEver 1990). P-selectin mediates the adherence of neutrophils and monocytes to platelets and endothelial cells via the sialyl-3-fucosyl-N-acetyl-lactosamine (SLe^x, CD155) (Larsen 1989, Handa 1991). In intact blood vessels, the interaction between neutrophils and P-selectin on endothelial cells leads to leucocytes rolling on the endothelium, the first step in leucocyte transmigration (Mayadas 1993). The role of P-selectin in platelets has not been fully defined but is thought to be important in the inflammatory response.

CD63 (LAMP-3)

This protein is present in both lysosomal and dense granule membranes. When platelets are activated, it fuses with platelet surface membrane, making it a useful marker for platelet activation (Nieuwenhuis 1987, Abrams 1991).

Table 1.2 Platelet membrane glycoproteins.

Glycoprotein	Molecular weight	Molecules on platelet surface	Function	Absent or diminished in
Ia/IIa	1a- 150,000	1,000	vWF-independent collagen-platelet interaction	
	IIa-138,000			
Ib/IX	Ib- 170,000	25,000	vWF and thrombin receptors, quinidine-antibody receptor	Bernard-Soulier syndrome
	IX- 17,000	25,000		
Ic/IIa	Ic- 140,000	1,000	Fibronectin receptor	
	IIa-138,000			
IIb/IIIa	IIb-145,000	40,000-80,000	vWF and fibrinogen receptors	Glanzmann's thrombasthenia
	IIIa-90,000			
IV	88,000	20,000	Thrombospondin and collagen receptors	
V	82,000	12,500	Thrombin and collagen receptors	Bernard-Soulier syndrome
VI	62,000	2,000	Collagen receptor	
P-selectin	140,000	2,600	Platelet-leucocyte adhesion	
LAMP-3	53,000	10,000*	Unknown	Hermansky-Pudlak syndrome

LAMP-lysosomal associated membrane proteins, * on lysosomal membrane

iii. Platelet energy generation

Platelet energy is derived from the metabolism of glucose, which is taken up from the plasma, and to a lesser extent from the metabolism of fatty acids. The energy is utilised for the maintenance of platelet structural integrity and the response of platelet to stimuli. The metabolism of carbohydrate in platelets is similar to that of other cells. About 80% of the glucose taken up by platelets is metabolised anaerobically (glycolysis), whereas one-fifth is carried through aerobically (oxidative phosphorylation). However, during glycolysis one mole of glucose gives a net yield of two moles of ATP, whereas during oxidative phosphorylation 38 moles of ATP are produced. There are different opinions about which is most important in the production of ATP for maintenance of platelet function, as each of the pathways alone can produce sufficient ATP in platelets under normal nutritional conditions (Holmsen 1985).

iv. Platelet function

Only the platelet functions important in haemostasis are described. Other functions including phagocytosis are not in the scope of this introduction.

a. Platelet shape change

Upon activation, platelets transformed from normal discoid shape to spiny spheres with several long, thin protrusions (filopodia, pseudopods). This process involves contraction of the cytoplasmic microfilaments, centralisation of organelles, actin polymerisation and bundling in the filopodia. Platelet shape change may facilitate platelet adhesion by the reduction of electrostatic repulsion, thus the end of the filopodia can approach and make contact with a surface or a cell.

b. Platelet adhesion

Platelet adhesion is defined as the process of platelets adhering to nonplatelet surfaces such as an artificial surface or subendothelial collagen/basement membrane.

Subendothelial collagen in injured blood vessels is the major site of platelet adhesion. This process requires vWF, which forms a ligand between GPIb and GPIIb/IIIa on platelet membrane and subendothelial collagen.

c. Platelet release reaction

During platelet shape change, platelet organelles gather in the centre of the cell. The membranes of the organelles then fuse with the membranes of the canalicular system, resulting in extrusion of the organelle contents into the canalicular system and to the extracellular environment. The release of ADP from platelet dense bodies is believed to be the key factor in the initiation of physiologic platelet aggregation. It induces the conformational change of GPIIb/IIIa, the major fibrinogen-binding site on the platelet membrane.

d. Platelet aggregation

Platelet aggregation is defined as the attachment of activated platelets to one another. The attachment of two platelets to one molecule of fibrinogen via GPIIb/IIIa is the initial step in platelet aggregation, which leads to the formation of a primary haemostatic plug. Once fibrin is formed, it reinforces the primary haemostatic plug which becomes a consolidated or secondary haemostatic plug.

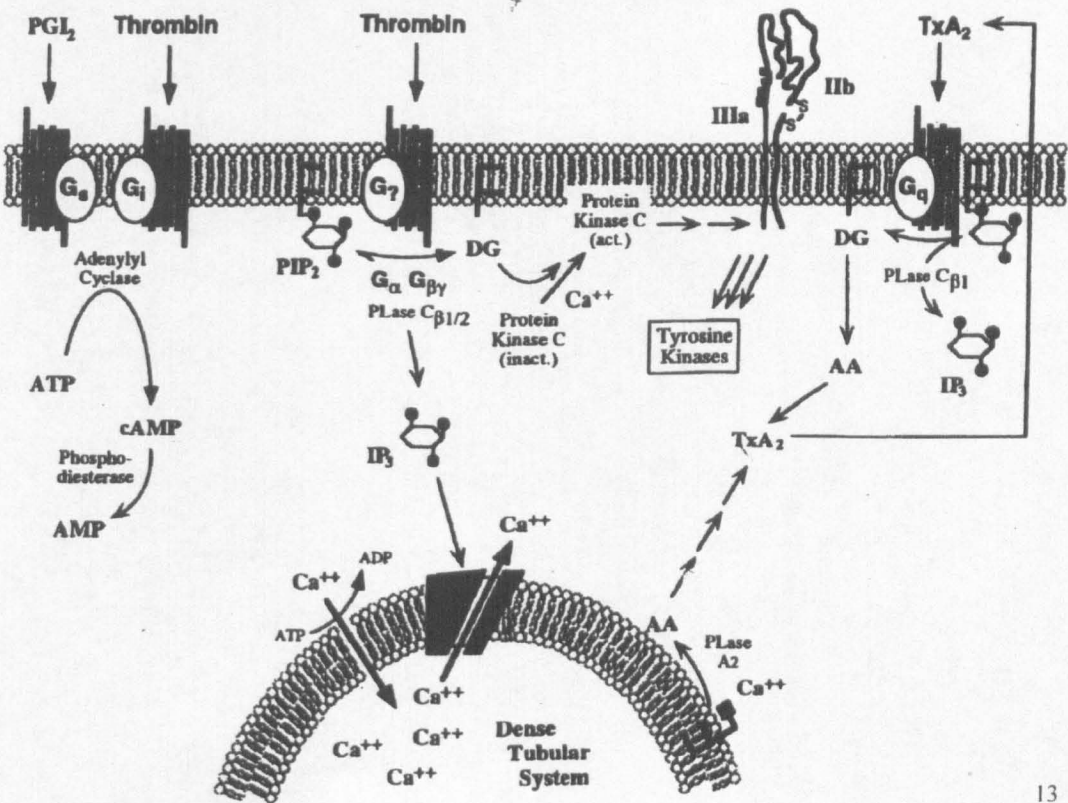
e. Signal transduction pathway and modulation of platelet function

Collagen and thrombin are primary activators at the site of vascular injury. The binding of platelet agonists to receptors on platelet surfaces generates signals. These signals are then transduced across the plasma membrane to appropriate effector enzymes, such as adenyl cyclase, phospholipase C, phospholipase A₂, via guanine nucleotide binding (G) proteins. Once the effector enzymes are activated, the second messengers, including inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DG) are generated. IP₃ releases Ca²⁺ from the dense tubular system, raising the cytosolic Ca²⁺ concentration. In turn, this promotes the activity of enzymes which do not function at the low Ca²⁺

concentration present in resting platelets. DG activates protein kinase C, triggering granule secretion and fibrinogen receptor exposure on GPIIb/IIIa. The rise in cytoplasmic Ca^{2+} also facilitates the release of arachidonic acid (AA) from membrane phospholipids by the action of phospholipase A_2 . This process may occur at both the plasma membrane and the membrane of dense tubular system. AA is then metabolised to thromboxane A_2 (TxA_2) which is a potent stimulus for platelet activation. TxA_2 can diffuse across the membrane and interacts with receptors on the platelet surface, resulting in further platelet activation. While the generation of second messengers promotes platelet aggregation, adenylyl cyclase activates the formation of cyclic AMP (cAMP) which inhibits platelet activation. (Figure 1.3)

Figure 1.3 Signal transduction during platelet activation.

(From Brass LF, Hoxie JA, Manning DR. Thrombosis and Haemostasis 1993;70:217-223.)



f. Platelet coagulant/anticoagulant activities

The negatively charged phospholipids in particular phosphatidylserine on the membrane of activated platelets act as a catalytic surface for at least two membrane-associated enzyme-substrate complexes, i.e. the formation of tenase (FVIIIa, FIXa, FX, Ca^{2+}), and prothrombinase complexes (FXa, FVa, prothrombin, Ca^{2+}) (Rosing 1988, Mann 1988, Schrott 1991, Zwaal 1996). It also enhances the activation of FX by tissue factor-FVIIa (Morrissey 1997). The presence of a catalytic membrane is essential for at least three reasons. Firstly, as the coagulation proteins circulate in plasma in relatively low concentrations in their zymogen (inactive) forms, a recruitment process is required to capture and concentrate the relevant subcomponents for optimal enzyme-substrate interactions. Secondly, the localised binding of the coagulation factors ensures that the amplification process does not propagate beyond the site of injury, resulting in pathological occlusion of vessels by thrombus. Finally, the membrane binding protects the activated coagulation proteins from inhibition by physiologic inhibitors due to steric hindrance. It is believed that the exposed phosphatidyl serine is the overall rate limiting step in the coagulation cascade in vivo.

Although less emphasis has been placed on the anticoagulant property of the same catalytic membrane, it should be noted that this membrane can also support the formation of the protein Case complex, one of the key components in the inhibition of coagulation pathway as described below.

1.1.3 Blood coagulation

Blood coagulation is an extremely complex process, involving several inter-related reactions. Numerous models have been proposed to explain this process consisting of: a successive step of activation of coagulation factors leading to the generation of thrombin and fibrin formation; inhibition where various physiologic inhibitors play the important role in limiting the clot formation to the site of injury and

neutralising active procoagulant factors that may enter the systemic circulation; and fibrinolysis where fibrin clot is digested after its haemostatic function has been fulfilled. The most recent concepts are discussed below.

i. Activation of coagulation factors

It is widely believed that tissue factor (TF, thromboplastin, CD142) is the most important physiological and pathological initiator of blood coagulation in vivo as shown in Figure 1.4. TF is an integral membrane protein found on the surface of a variety of cell types normally located outside the vasculature and exposed as a consequence of vascular damage from mechanical injury or inflammatory process.

In normal circulation, approximately 1 to 2% of FVII molecules are present in the form of FVIIa. Free FVIIa does not express enzymatic activity. However, when FVIIa binds to TF, it can convert FX to FXa. FXa can generate a small amount of thrombin but once produced, thrombin together with the initially formed FXa activates FV to FVa and FVIII to FVIIIa. FXa-TF-FVIIa complex can also activate FIX to FIXa. FIXa-FVIIIa (intrinsic tenase) converts FX to FXa, and FXa-FVa (prothrombinase) converts prothrombin to thrombin. The production of FXa by FIXa-FVIIIa is 50-fold more efficient than FVIIa-TF. In addition, tissue factor pathway inhibitor (TFPI) binds to FXa-TF-FVIIa to limit the production of FIXa and FXa by this pathway. Once this inhibitory reaction occurs, FXa can only be produced by the FIXa-FVIIIa complex. The importance of these two molecules is demonstrated by the pathology associated with their abnormalities in vivo (Davie 1995, Mann 1999, Morrissey 1997, Nemerson 1996)

The initiation of blood coagulation through the activation of FXII, plasma prekallikrein (PK) and high molecular weight kininogen (HMWK) (contact system) is an important pathway in the presence of artificial surfaces (Colman 1999, Mann 1999, Schmaier 1997, 1999). However, the initiation of coagulation through this system is believed to be less important in vivo than TF as the deficiency of factors involved in the

contact system is not associated with bleeding disorders. The deficiency of FXI is associated with bleeding, but the pathology is mild.

In the presence of artificial negatively charged surfaces such as glass, kaolin, insoluble metal complexes of elagic acid, dextran sulphate, sulfatides, or phospholipids, the binding of FXII leads to the autoactivation of FXII to FXIIa (Silverberg 1980) as shown in Figure 1.5. In addition, PK and FXI, which circulate in plasma as a complex to HMWK, also bind to these surfaces. FXIIa activates PK to kallikrein, then kallikrein reciprocally activates more FXII in a reaction that is at least 1,000-fold faster than autoactivation. The rate of initiation and amplification of this system is accelerated by HMWK. Kallikrein as well as FXIIa releases bradykinin (BK) from HMWK. FXIIa and kallikrein activate FXI to FXIa which activates FIX to FIXa. In the presence of activated platelets, FXI can also be activated by thrombin. Together with FVIIIa, FIXa forms an intrinsic tenase leading to thrombin formation. FXIIa can also activate FVII.

In the absence of negatively charged artificial surfaces, it has been proposed that the assembly of the contact factors on membranes of cells in the intravascular compartment such as platelets, endothelial cells, neutrophils, etc., triggers the activation of this system. More recently, it has been proposed that binding of PK complexed to HMWK on endothelial cells can generate kallikrein and BK by endothelial cell cysteine protease, independent of FXII activation (Motta 1998, Rojkaer 1998, Schmaier 1997, 1998, 1999).

The role of the contact system in antithrombosis and profibrinolysis is believed to be more important than in haemostasis. Kallikrein and FXIIa cleave plasminogen directly although the reaction is slower than tissue-plasminogen activator or urokinase plasminogen activator. Kininogens inhibit platelet activation by α -thrombin. Bradykinin is a potent stimulator of prostacyclin synthesis in the endothelial cell. Prostacyclin stimulates adenylylate cyclase leading to increased intracellular cAMP, which inhibits

platelet function. Bradykinin also stimulates nitric oxide formation, which then stimulates guanylate cyclase leading to the elevation of cGMP and inhibition of platelet function. Furthermore bradykinin stimulates tissue plasminogen activator secretion, enhancing fibrinolysis (Schmaier 1997, 1998, 1999).

Apart from the coagulation system, FXIIa, kininogens and kallikrein also contribute to the activation of the first complement component (C1), and granulocytes. Furthermore, FXII downregulates monocyte Fc receptors, and releases IL-1 and IL-6 from monocytes and macrophages.

The formation of fibrin begins when thrombin cleaves fibrinopeptides A and B from fibrinogen, leaving fibrin monomer which aggregates to form soluble fibrin. Thrombin also activates FXIII to FXIIIa which converts soluble fibrin to insoluble fibrin by cross-linking .

Figure 1.4 The activation of coagulation cascade by tissue factor pathway.

(From Davie EW, Fujikawa K, Kiesel W. Biochemistry 1991;30:10364-10370.)

PL phospholipid

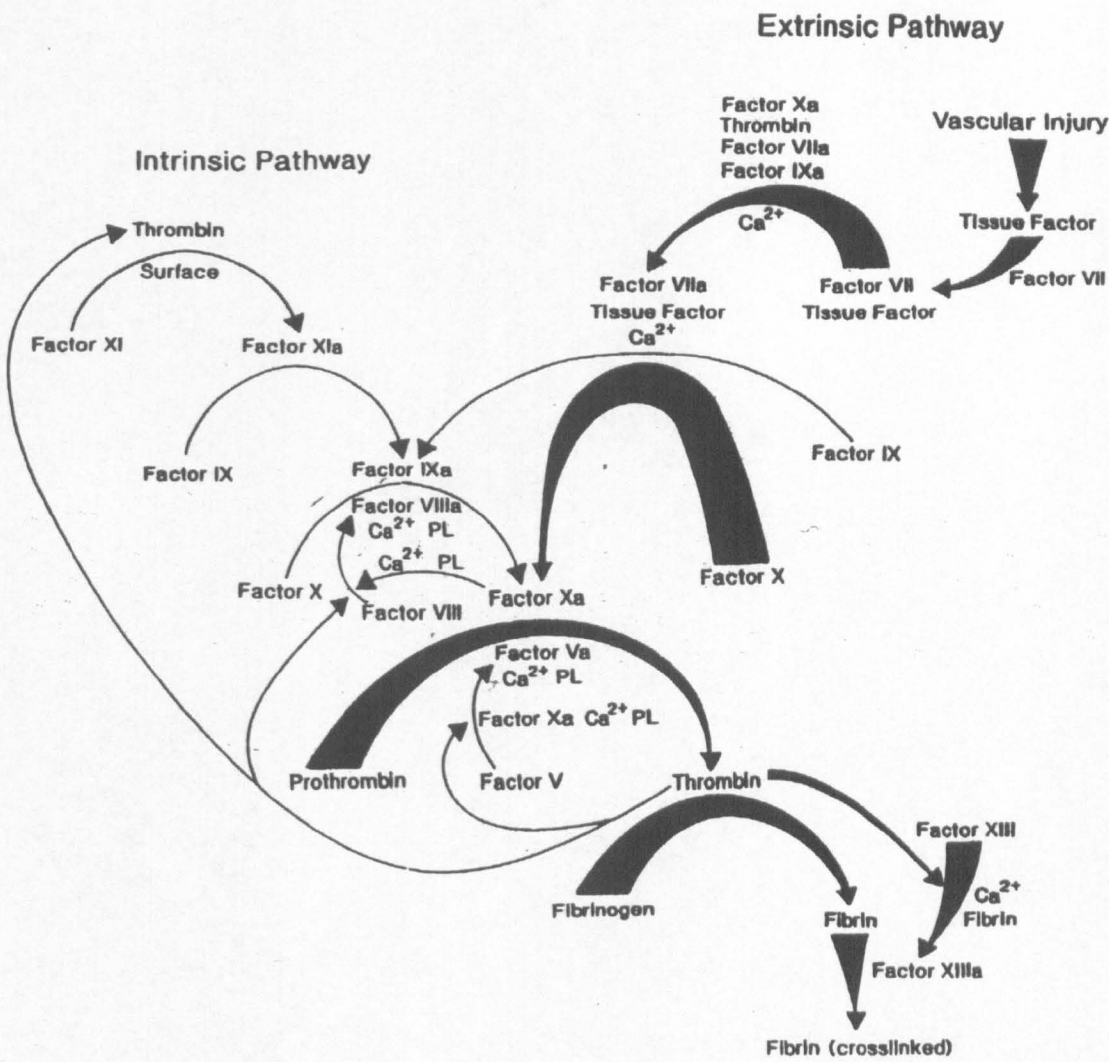
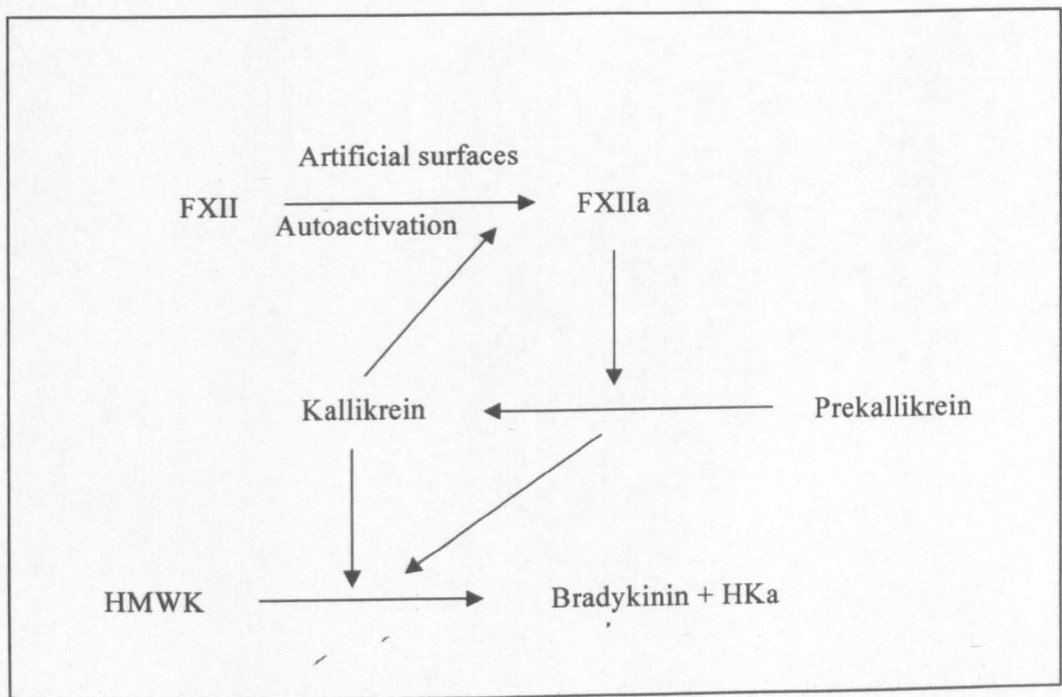


Figure 1.5 The activation of the contact system on artificial surfaces.



ii. Inhibition of coagulation

Similarly to other biological processes, the blood coagulation system is regulated by a number of inhibitory mechanisms designed to restrict clotting to a given site. Apart from TFPI which is formed when TF pathway is activated, there are several other inhibitors, referred to as serpins, containing broad spectrum serine protease inhibitors. The main inhibitors with their neutralising capacity are summarised in Table 1.3. The most three important inhibitors, antithrombin III (ATIII), protein C and protein S, are also described below.

Table 1.3. Plasma serine protease inhibitors and their neutralising capacity.

	<i>Inhibitors</i>							
	ATIII	HCII	C1inh	α_1 AT	α_2 M	α_2 AP	PCI	TFPI
<i>Target enzymes</i>								
FIIa	+	+	-	+	+	+	+	-
FVIIa	-	-	-	-	-	-	-	+
FIXa	+	-	-	-	-	-	-	-
FXa	+	+	-	+	+	+	+	+
FXIa	+	-	+	+	-	+	+	-
FXIIa	+	-	+	-	-	+	-	-
Kallikrein	+	-	+	-	+	-	+	-
APC	-	-	+	+	-	+	+	-
tPA	-	-	-	-	-	-	+	-
Plasmin	+	-	+	-	+	+	-	-

APC-Activated protein C, tPA-Tissue plasminogen inhibitor, ATIII-Antithrombin III, HCII-Heparin cofactor II, C1inh-C1 inhibitor, α_1 AT α_1 -antitrypsin, α_2 M- α_2 -macroglobulin, α_2 AP- α_2 -antiplasmin, PCI-Protein C inhibitor, TFPI-Tissue factor pathway inhibitor

Antithrombin III binds irreversibly with the serine proteases, thrombin, FIXa, FXa, FXIa, FXIIa and kallikrein, and forms complexes which are then removed from the circulation. The inhibitory effect of ATIII is markedly enhanced in the presence of heparin.

Two interrelated pathways of the inhibitory mechanism of protein C have been described, both of which require a catalytic surface. Thrombin binds to thrombomodulin on endothelium and converts protein C to activated protein C (APC). Activated protein C then inactivates FVa leading to the termination of prothrombinase activity. APC can also inactivate FVIIIa but from a physiological perspective this is thought to be irrelevant due

to the instability of FVIIIa. Another mechanism of anticoagulant activity of protein C is through the formation of membrane-associated enzyme-substrate complex (protein C complex) consisting of thrombin, thrombomodulin, protein S, and protein C. The activity of APC is, in turn, regulated by two major serine protease inhibitors, protein C inhibitor and α 1-antitrypsin, and also by C1 inhibitor and α 2-antiplasmin.

ii. Fibrinolysis

The fibrinolytic system is responsible for the destruction of a fibrin clot after its haemostatic function has been fulfilled. This system consists of plasminogen (proenzyme) which can be converted to plasmin (active enzyme) by several pathways. Two primary plasminogen activators in physiological conditions are tissue plasminogen activator (tPA) and urokinase-like plasminogen activator. Plasmin can also be generated by direct/indirect activation by FXIIa and kallikrein (Rojkjaer 1998). Unlike thrombin which has a very narrow substrate specificity, plasmin has a much broader spectrum of activity with a number of substrates. Plasmin biodegrades fibrinogen and fibrin into fibrin(ogen)-degradation products (FDPs). Plasmin also degrades FV, FVIII, FIX and FXI. Plasmin exists in both free and fibrin-adsorbed forms. Free plasmin is rapidly destroyed by antiplasmin. This confines the fibrinolytic activity to the site that it is required in physiologic conditions i.e. within the fibrin mass. The binding of plasmin to fibrin blocks the antiplasmin binding sites and protects plasmin from inactivation.

The fibrinolytic system is regulated by several inhibitors (Table 1.3). The major inhibitors of plasmin are α 2-antiplasmin (plasmin inhibitor) and α 2-macroglobulin. The inhibitors of tPA are tissue plasminogen activator inhibitors 1 and 2 (tPAI-1 and tPAI-2).

1.1.4 Complement system

Similar to the coagulation cascade, the complement system is activated through a cascade of proteolytic cleavage of its earlier components. Upon activation the complement proteins can give rise to mediators of chemotaxis, increased vascular

permeability, opsonic activity, phagocytic activation, and cytolysis (Table 1.4). The activation of complement system can occur via two pathways, classical and alternative as summarised in Figure 1.6. The classical pathway is initiated by complement-fixing immune complexes (IgG and IgM), while the alternative pathway is triggered by a variety of substances including IgA aggregates, endotoxin, and bacterial cell wall polysaccharides.

The classical pathway proceeds by fixing C1 (C1q,r,s) with the Fc portion of IgG or IgM. The activated C1 then cleaves C2 and C4 to form C3 convertase (C4b2a). The alternative pathway, however, cleaves C3 to C3a and C3b directly. C3b binds to factor B and D and is stabilised by properdin to form C3 convertase (C3bBbP). C3 convertase generated by either pathway cleaves C3. C3 convertase binds to C3b to form C5 convertase which cleaves C5 into C5a and C5b. C5b is then binds to C6, C7, C8 and C9 to form C5b-9, the membrane attack complex. (McAleer 1993, Meri 1998, Mollnes 1998)

Figure 1.6 The activation of complement system.

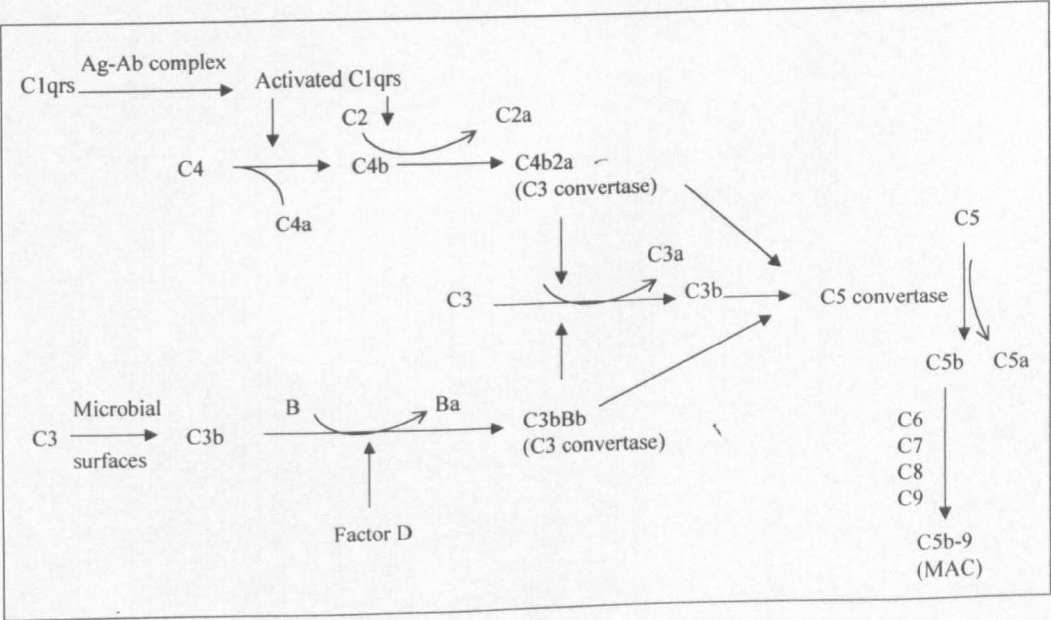


Table 1. 4 Proinflammatory complement components.

Complement component	Biological activity
C3a (anaphylatoxin)	Increases vascular permeability
C5a (anaphylatoxin)	Increases vascular permeability, chemotaxis and activation of granulocytes
C3b	Opsonises particulates e.g. bacteria, and binds to phagocytes
C5b-9 (membrane attack complex)	Cytolysis, trigger cytokine, prostaglandin release, and oxidant production by monocytes

The complement system is regulated by three control systems: direct inhibition of serine proteases, decay and destruction of convertases, and control of the membrane attack complex. Table 1.5 describes the regulation of the complement system.

Table 1.5 Control of the complement system.

Inhibition of serine protease

C1 inhibitor dissociates C1r and C1s from C1q-activated complex.

Decay and destruction of convertases

C2a and Bb are dissociated from C3a convertase and C5a convertase

C4b-binding protein, factor I, factor H, complement receptor type 1 (CR1) and decay accelerating factor (DAF) bind to C3a convertase and C5a convertase causing dissociation of the proteins with decay accelerating activity.

Control of membrane attack complex

S-protein (vitronectin), SP40, and MAC-inhibiting protein (MIP) inhibit MAC formation.

CD59 (protectin, homologous restriction factor HRF 20) inhibits cell lysis by preventing channel formation in the membrane.

1.2 PLATELET CONCENTRATES FOR TRANSFUSION

1.2.1 Preparation of platelet concentrates

Platelets for transfusion have been prepared since the 1960s (Aster 1965). The separation of platelets from whole blood is based on differential centrifugation as various cellular components of blood i.e. red blood cells, leucocytes and platelets have different specific gravity, and hence a different sedimentation velocity. Three main procedures for harvesting platelets for transfusion are currently in use. The first two use whole blood donations of 450 ± 45 mL as the start material, while the third harvests platelets and return other blood elements to the donor.

i. Platelet rich plasma method

In this method, platelets are separated from whole blood by two-stage differential centrifugation. In the first stage, a "soft spin" (e.g. up to 1000 g, 2-4 minutes) is used to separate platelet rich plasma containing the majority of platelets and 30 to 50% of leucocytes as well as some red blood cells, from the majority of red blood cells. Platelet rich plasma is then transferred to a satellite bag and platelets are pelleted in the second stage using a "hard spin" (e.g. 2000-3000 g, 7-10 minutes). This is the routine method in North America. The high centrifugation g force used to pellet platelets against the bottom of the bag is highly traumatic to platelets. This leads to high levels of platelet activation and aggregation, as compared to platelets prepared by buffy coat method described below (Fijnheer 1990, Metcalfe 1997).

ii. Buffy coat method

This is the routine method in the UK. This method uses a special bag system, "bottom and top", and a "hard spin" (e.g. 2,000 to 3,000 g, 7 to 10 minutes) to separate buffy coat from plasma and red blood cells (Figure 1.7). The buffy coat obtained contains approximately 70 to 80% of the platelets and leucocytes, and 10% of the red blood cells

present in a unit of whole blood. Platelets can be prepared from either a single unit or a pool of 4 to 6 units of buffy coats with plasma added. Platelets are harvested using a "soft spin" (e.g. 700 to 1000 g, 2-5 minutes). As the pelleting process is performed on a cushion of leucocytes and red blood cells, platelets prepared from buffy coat method are less activated than platelet rich plasma method (Fijnheer 1990, Metcalfe 1997).

iii. Plateletpheresis

Using automated apheresis machines, after platelets have been separated from whole blood, red cells and some plasma are either returned to the donor or collected as separate components. Although the underlying principle is still based on differential centrifugation, the final product from one donor contains sufficient number of platelets for at least one therapeutic dose i.e. 240×10^9 platelets. Furthermore, with some new generation machines, double or even triple doses can be collected from a single donation by selecting donors with a certain pre-donation platelet count. In addition, almost all of the modern apheresis machines provide leucocyte depleted products i.e. less than 5×10^6 leucocytes per unit of one therapeutic dose of platelets, either with or without the use of a leucocyte removal filter.

Three major techniques for plateletpheresis are employed by various manufacturers:

a. Buffy coat technique

This technique is used in the Haemonetics MCS+ apheresis machine (Haemonetics, Baintree, MA, USA) (Haemonetics Manual) (Figure 1.8). Anticoagulated whole blood is pumped into a spinning "Latham" bowl and separated into red cells, buffy coat and plasma layers. Once the plasma separation is detected by the optical sensor, plasma is pumped into a plasma bag with some recirculating into the bowl to dilute the buffy coat layer for a better separation of platelets (dwell phase). The dwell phase is followed by the surge phase where plasma is pumped out of the bowl passing an optical

sensor and the light transmission is recorded as 100%. As platelets start to exit the bowl, the light transmission decreases and when it reaches the threshold setting i.e. 87%, the collection of platelets starts. The sensor continues to monitor the light transmission, and once the maximal number of platelets have exited the bowl the light transmission will start to increase which terminates platelet collection. Red cells which remain in the bowl are then returned to the donor with plasma before the next cycle begins. With the modification of software using the "LD" collection protocol, filtration of platelet concentrates can be carried out automatically during the last return cycle or during each return cycle (Holme 1999).

b. Platelet rich plasma combined with fluidised particle-bed

This technique is used in the COBE Spectra Leucoreduction System [LRS] (COBE BCT, Lakewood, CO, USA) to provide leucocyte depleted platelet concentrates without leucocyte filtration (Adams 1998) (Figure 1.9). The machine consists of a polyester conical shape chamber (LRS chamber) connected to a rotating blood collection set. Firstly, platelet rich plasma is separated from whole blood in the blood collection set. Secondly, the platelet rich plasma is pumped into the rotating LRS chamber. As flow begins, the plasma flow velocity through the chamber is directed radially inward, whereas the platelet flow velocity is directed radially outward. Platelets start to sediment in the chamber and continue to accumulate to create a saturated platelet bed. At saturation, the interstitial plasma velocity between platelets is slightly greater than the plasma sedimentation velocity, and the flow past any single platelet is just large enough to lift the platelet slowly out of the gravitation field. The leucocytes that enter the chamber with platelets sediment much faster than platelets and remain trapped below (radially outward from) the platelet bed in the chamber.

c. Platelet rich plasma combined with elutriation

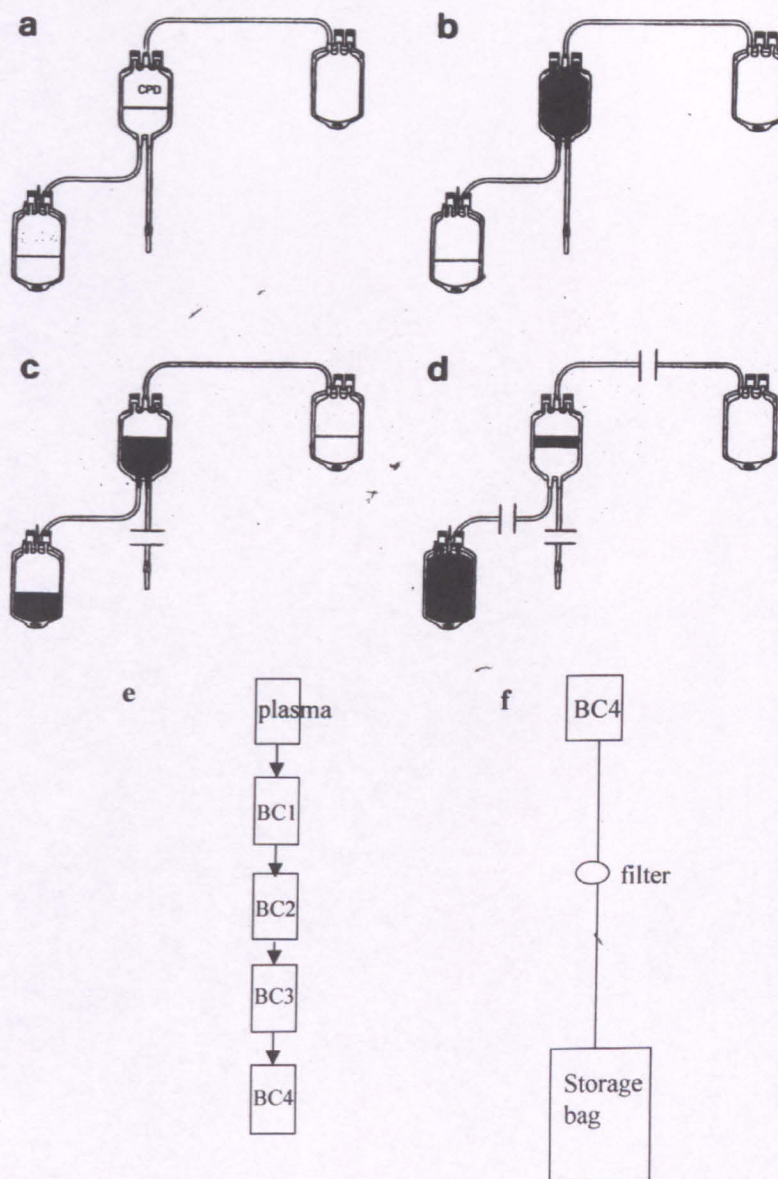
This technique is employed by the Amicus apheresis machine (Baxter Healthcare,

Illinois, USA) and provides leucocyte depleted platelets without the use of a leucocyte removal filter (Burgstaler 1997) (Figure 1.10). The system contains separation and collection containers connected like a belt by a multilumen tube. Anticoagulated whole blood is drawn into the separation chamber where platelet rich plasma is separated from red cells and most of the leucocytes by centrifugation. Inside the centrifuge is an interface detector which keeps the interface separation constant throughout the whole procedure. The platelet rich plasma is pumped into the collection chamber where platelets are concentrated; some of the platelet rich plasma is recycled back to the incoming whole blood to reduce the haematocrit prior to entering the separation chamber. As the reduced haematocrit blood enters the separation chamber, the platelet rich plasma exits quickly causing an elutriation effect, pushing large platelets on the interface out of the separation chamber at the same end where the whole blood enters.

At the same time as platelet rich plasma is drawn out of the separation chamber, granulocytes, cells with relatively high specific gravity, drop to the bottom of the chamber and exit with red blood cells to be returned to the donor. Mononuclear cells are drawn off with platelet rich plasma but when they encounter more dilute red blood cells they drop back down again; hence they loop around the far end of the chamber.

Figure 1.7 Preparation of platelet concentrates by buffy coat method.

a. Configuration of the "bottom and top" bag system. b. Whole blood is collected from the lower tubing into the primary bag containing CPD anticoagulant. c. After centrifugation, the plasma is transferred into the upper transfer bag, while the red cells are pressed simultaneously into the lower transfer bag. d. The buffy coat remains in the primary bag. e. One unit of plasma and four units of buffy coats pooled into one of the buffy coat bags (BC4). f. The bag containing pooled buffy coats is connected to an in-process leucocyte removal filter attached to a platelet storage bag. After centrifugation, the platelet rich plasma is passed through the filter into the storage bag.



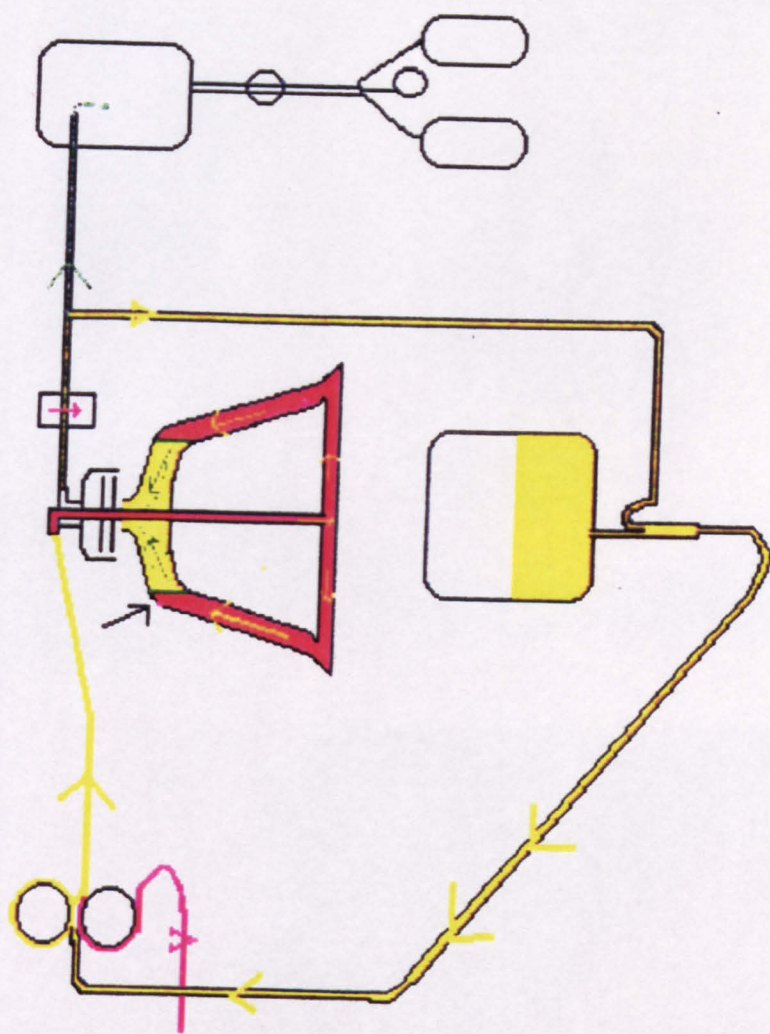


Figure 1.8 Platelet collection by Haemonetics MCS+.

In the spinning "Latham" bowl, anticoagulated whole blood is separated into red cell (red), buffy coat (black arrow), and plasma (yellow) layers.

Plasma containing platelets (green) is pumped out of the bowl passing a sensor (pink arrow) into a collection bag.

Figure 1.9 Platelet collection by Cobe LRS.

In the blood collection set (top) platelet rich plasma is separated from whole blood and pumped into the spinning LRS chamber (bottom). Leucocytes are trapped below the platelet bed in the chamber.

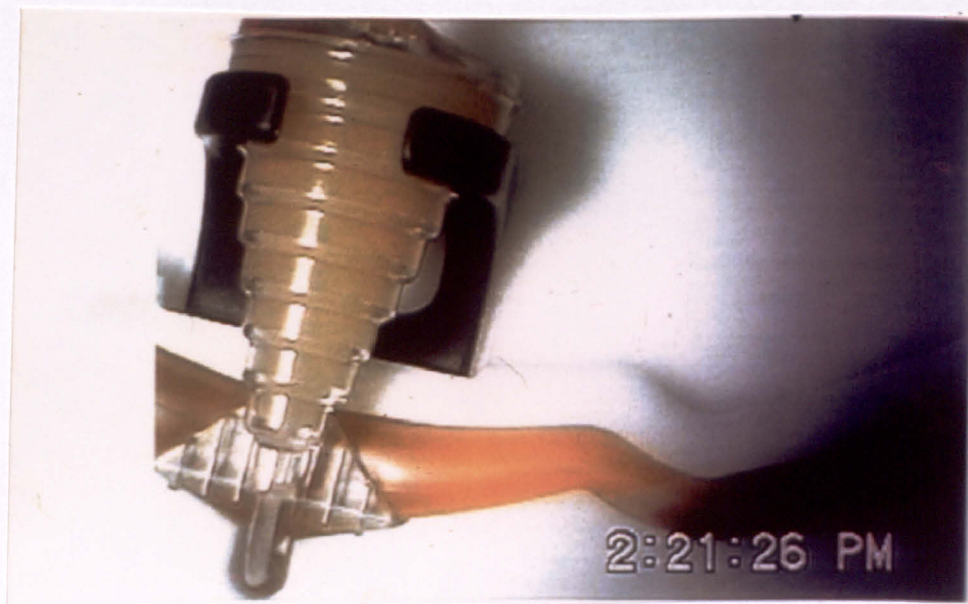
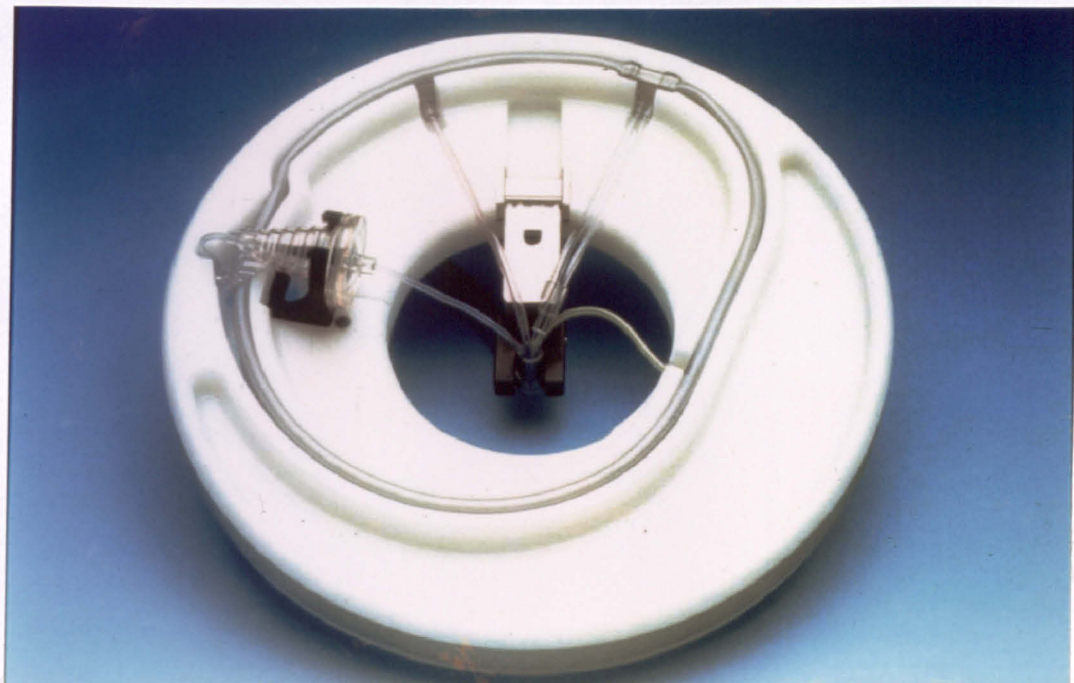
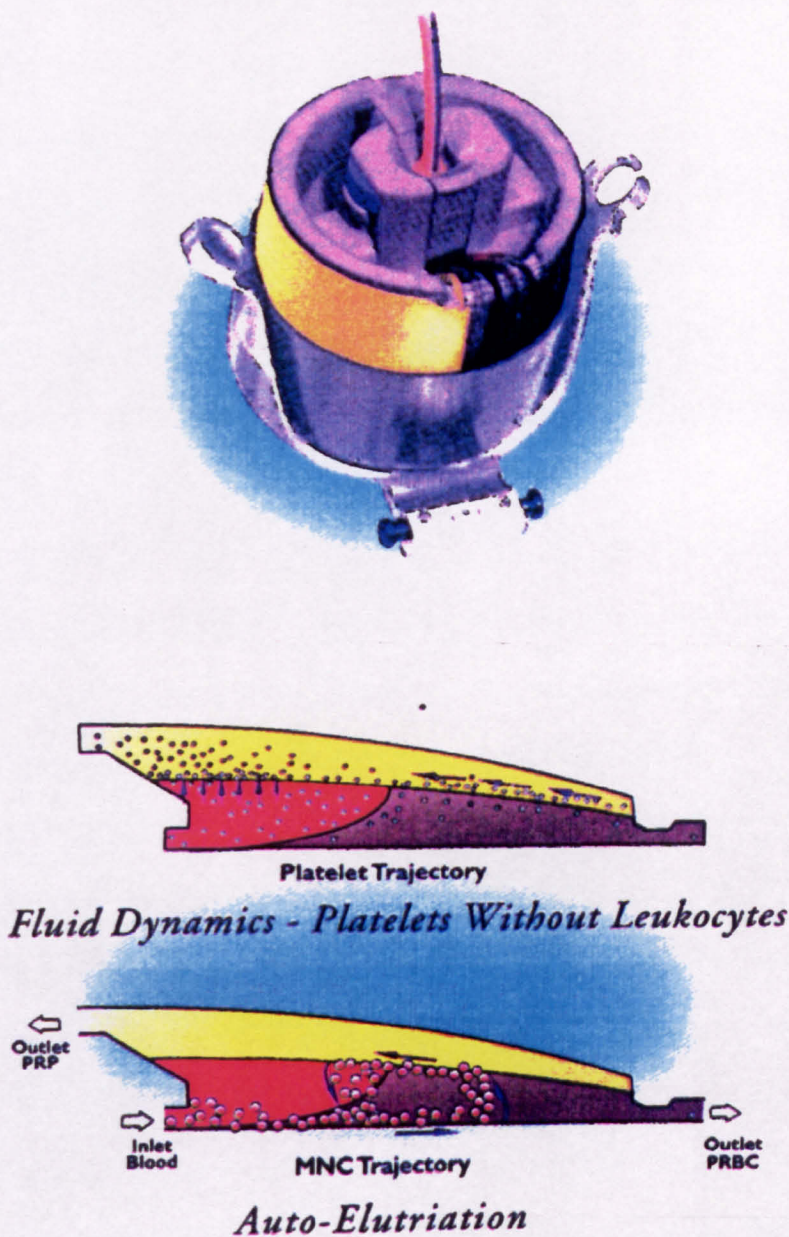


Figure 1.10 Platelet collection by Amicus.

The separation and collection chambers are connected like a belt (top). In the separation chamber (bottom, cross section), platelet rich plasma (yellow) is separated from red cells (red) and most of the leucocytes (round particles). The exit of platelet rich plasma causes an elutriation effect making leucocytes drop to the bottom of the chamber.



1.2.2 Storage of platelet concentrates

Currently platelet concentrates for clinical use can be stored up to 5 days and still provide effective haemostatic function in vivo. However, they must be stored in optimal conditions influenced by the following:

i. Storage bags

To maintain their physiologic function platelets are required to be stored in a container made from biocompatible materials which allow oxygen/carbon dioxide exchange. For this purpose polyvinylchloride (PVC), a polymer, in combination with various plasticisers has been used. PVC, when unplasticised, is rigid. Plasticisers are liquids that dissolve in a polymer. The resultant mixture is a solution of a liquid in a solid. This lowers the attraction between the polymer chains and allows them to slide by each other. In effect, a plasticiser reduces the crystallinity of polymer, hence increasing the flexibility.

Several plasticisers are currently in used in platelet storage bags. These are diethylhexylphthalate (DEHP, sometimes referred to as dioctylphthalate or DOP), trioctyl trimellitate (TOTM, sometimes referred to as triethylhexyl trimellitate or TEHTM), butyryl tri-n-hexyl citrate (BTHC), and di-n-decylphthalate (DnDP).

Plasticisers are highly soluble in lipids. Various amounts of plasticisers, depending on their solubility characteristics, are leached into plasma and some are incorporated into the platelet membrane (Fratantoni 1992). Although significant amounts of plasticisers are transfused into patients receiving platelet concentrates, they are far below the toxic dose. More important are their effects on platelet function and membrane integrity. The partition of plasticisers into platelet membrane has been shown to enhance membrane stabilisation and reduce platelet responses to stimuli measured by shape change and aggregation (Ishikawa 1984, Shimizu 1989). However, it is unclear whether the reported findings were solely due to the effect of plasticisers or also the results of

other variables i.e. oxygen content and pH. Furthermore, it is not known whether such effects are beneficial or harmful. On the one hand, the stabilisation of the platelet membrane reduces changes occurring during storage. On the other hand, the decrease of platelet response to stimuli may affect in vivo haemostatic effectiveness.

More recently, platelet storage bag made from modified polyolefin has become available. Unlike PVC, it does not require plasticiser.

ii. Agitators

Continual gentle agitation is essential during the storage of platelet concentrates to facilitate gas exchange within the bag and reduce the formation of platelet aggregates. However, agitation may also stimulate platelets through interaction with the bag wall. Three kinds of agitators have been designed: flatbed (tumbler, horizontal) 90 cycles per minute, circular (end-over-end) 2 rpm, and elliptical (side-over-side) 6 rpm agitators. The importance of the type of agitators during storage has been highlighted by a study comparing changes in platelet concentrates kept on flatbed vs elliptical agitators. Platelets stored in the latter rotator showed higher levels of P-selectin, greater loss of GPIb and higher level of microvesicles (George 1992). This observation was attributed to the mixing features of the agitators used in the study. In the flatbed agitator the platelet bags were face up or face down, with no trapping of platelets in the folds or edges of the bag, achieving thorough mixing of the platelet suspension. In the elliptical agitator the bags were placed edge up and edge down, and an end of the bag had to be folded to hold it in a clip of the rotator. This design led to trapping of some platelets into the folded edges and corners of the bag, and hence incomplete mixing and aggregate formation.

iii. Storage temperature

Platelet concentrates were routinely stored at 2 to 4°C in the same manner as red cell concentrates until 1969 when it was shown that the viability of refrigerated platelets was less than half that of platelets kept at 22°C (Murphy 1969). The mechanism of the

shortened life-span of cold stored platelets is unknown but appears to be related to cold-induced discoid-spheric transformation, loss of platelet membrane GPIb and microvesiculation (Bode 1994).

iii. Platelet storage media

Traditionally, platelets are suspended and stored in plasma. Platelet storage media (PSM) or platelet additive solutions (PAS) were developed to increase plasma availability for plasma protein fractionation, in particular to manufacture more FVIII used for haemophiliacs. The use of platelet storage media may also have some other potential benefits such as reducing the possible adverse reactions associated with plasma proteins and improving platelet quality by providing better nutritional supply (Adams 1986, Eriksson 1990, Holme 1987, 1992, Bertolini 1992a,b, Shimizu 1992, Gullikson 1993, 1995, Murphy 1995). The composition of platelet storage media is variable as shown in Table 1.6.

Table 1.6 **Composition of platelet storage media.**

Concentration in mmol/L	Plasma Lyte A (Rock 1985)	PAS (Holme 1987)	PSM1 (Murphy 1991)	Setosol (Shimizu 1992)	PAS-1 Gulliksson (1993)	PAS-3 Corash (1997)
NaCl	90.0	110.0	98.0	90.0	115.5	77.0
KCl	5.0	5.1	5.0	5.0	-	-
CaCl ₂	-	1.7	-	-	-	-
MgCl ₂	3.0	-	-	3.0	-	-
MgSO ₄	-	0.8	-	-	-	-
Na ₃ citrate	-	15.2	23.0	17.0	10.0	12.3
Citric acid	-	2.7	-	-	-	-
NaHCO ₃	-	35.0	-	-	-	-
Na phosphate	-	2.7	25.0	25.0	-	28.0
Na acetate	27.0	-	-	23.0	30.0	42.0
Na gluconate	23.0	-	-	-	-	-
Glucose	-	35.5	-	23.5	-	-
Maltose	-	-	-	28.8	-	-

PAS-platelet additive solution, PSM-platelet storage media

The presence of glucose creates manufacturing problems because of its caramelisation during sterilisation at neutral pH. Consequently, the recent practice has been to not include glucose and use acetate as an alternative fuel. Acetate also provides buffering effect as it requires hydrogen ion to convert to acetyl CoA (Figure 1.10). The need for glucose in the storage media is debated because it contributes only approximately 15% of ATP regeneration. Nevertheless, it may be required for the activity of the hexose monophosphate shunt and for the provision of ATP for specific membrane functions such as Na^+/K^+ exchange (Paul 1983).

To date studies have shown that approximately 30% of plasma is required with the use of glucose-free storage media to provide an optimal storage condition (Murphy 1999). The benefit of the carryover plasma might be due to that it provides glucose as well as some proteins such as albumin that may play a role in maintaining the structural integrity of platelets.

1.3 LEUCOCYTE DEPLETION OF PLATELET CONCENTRATES

According to the Guidelines for the Blood Transfusion Services in the UK, leucocyte depleted blood products contain less than 5×10^6 leucocytes per unit of adult therapeutic dose. The use of leucocyte-depleted platelet concentrates has been associated with a number of clinical advantages e.g. reduction in HLA alloimmunisation, transmission of cell-associated viruses such as cytomegalovirus, and alloimmunisation (Bordin 1994). Since November 1999, all blood components in the UK have been subjected to a leucocyte depletion step, as a precaution against the theoretical possibility of variant Creutzfeldt-Jacob disease (vCJD) transmission. This is because of evidence that the infectivity may be leucocyte associated (Turner 1999).

1.3.1 Techniques available

Currently leucocyte depleted platelet concentrates can be prepared by four major techniques:

- i. Leucocyte filtration of platelets derived from whole blood units
- ii. Leucocyte filtration of platelets collected by apheresis i.e. Haemonetics

MCS+ LD

- iii. Fluidised particle bed technology using Cobe Spectra LRS apheresis machine
- iv. Elutriation using Amicus apheresis machine

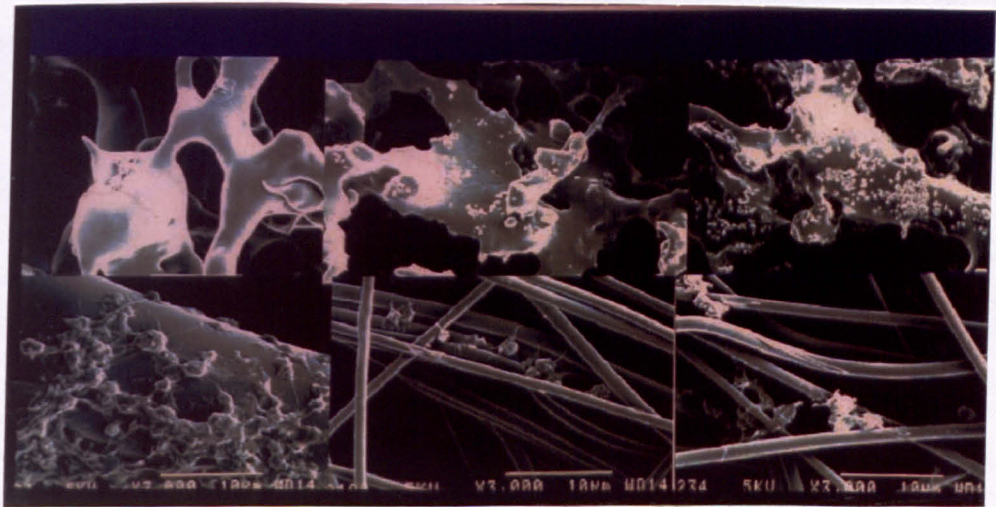
1.3.2 Mechanisms of leucocyte removal by filters

Modern leucocyte removal filters for platelet concentrates are made of two major types of synthetic materials, i.e. polyester and polyurethane. While polyester microfibres require surface charge modification to increase selective leucocyte removal and to decrease platelet binding to the filter (Kickler 1989, Cenni 1993), polyurethane, due to its microdomain structure, does not (Branwood 1994). Furthermore, the structure of filters made from polyester fibres is described as a mesh of nonwoven fibres, whereas those made from polyurethane are described as multiple layers of sponge-like porous sheet. The summary of the characteristic of major leucocyte removal filters is shown in Table 1.7. Scanning electron micrographs of polyester and polyurethane filters are shown in Figure 1.11.

Table 1.7 Synthetic material and structure of three commonly used white cell removal filters for platelet concentrates.

Filter	Manufacturer	Synthetic material	Structure
Autostop BC	Pall Biomedical	negatively charged polyester	mesh of nonwoven fibres
Sepacell PLX5	Asahi Medical	positively charged polyester	mesh of nonwoven fibres
Imugard III	Terumo	neutrally charged polyurethane	layers of sponge-like porous sheet

Figure 1.11 Scanning electron micrographs of polyester and polyurethane filters.
Top panels-polyurethane, bottom-polyester. (x3000)



While the mechanisms by which leucocytes are retained by filters for red cell products have been studied (Steneker 1992, Dzik 1993), the mechanisms involved in filters for platelet concentrates are not precisely known and some of the mechanisms described in the former are not applicable to the latter. Nevertheless, it is believed that the mechanisms are multifactorial and include:

i. Sieving or mechanical retention

This mechanism is based on size exclusion as platelets are smaller than leucocytes. The efficiency of this process involved several factors. These include cell deformability, cell concentration, and composition of blood product, as well as filter fibre diameter, density, total surface area, surface tension, pore diameter and pore size distribution (Dzik 1993).

ii. Cell adhesion

Leucocytes can adhere to filter materials by several mechanisms, i.e. direct binding; indirect binding mediated by leucocyte adhesion molecules (such as $\beta 2$ integrins) and/or pre-adsorbed plasma proteins (such as immunoglobulin, fibronectin); and interaction with platelets via P-selectin (Dzik 1993).

1.3.3 Effect of leucocyte removal filters for platelets on blood physiology

The contact of platelets and plasma proteins with filter materials can lead to a number of biological interactions:

i. Interaction with platelets

Several studies have shown that leucocyte filtration using various filters does not cause platelet activation (Bertolini 1990, Pedigo 1993, Metcalfe 1997). However, it is also possible that activated platelets expressing P-selectin are retained by the filters through the interaction with leucocytes.

ii. Generation or removal of microvesicles

During leucocyte filtration, platelets are subjected to shear stress. Studies have shown that shear stress can induce platelet activation and microvesiculation (Sakariassen 1998). On the other hand, it is possible that the pre-formed microvesicles can be removed by filter through the interaction between microvesicles expressing P-selectin and leucocytes. It is not known whether the final filtered products contain higher or lower numbers of microvesicles than pre-filtered counterparts.

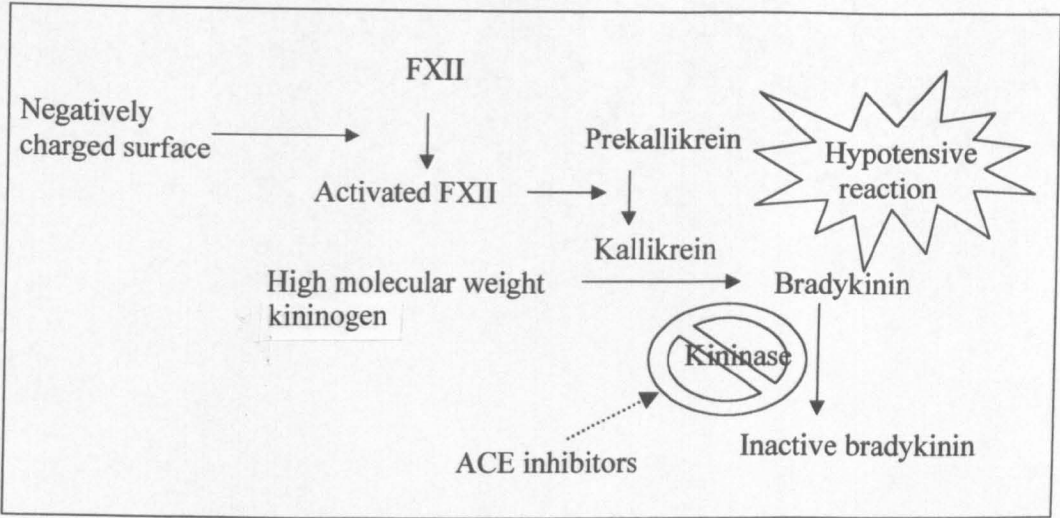
iii. Activation of the contact system of coagulation

The interaction between FXII and the artificial surface of filters, in particular those with a net negative charge, leads to the activation of the contact system resulting in the generation of bradykinin, a potent vasodilatory peptide. This interaction is reportedly associated with hypotensive reactions in transfused patients also receiving angiotensin converting enzyme (ACE) inhibitors (Hume 1996, Sano 1996, Mair 1998). Normally, bradykinin in plasma is degraded very rapidly (half-life 15 seconds) by kininase I and II. Kininase II (angiotensin converting enzyme) is inhibited by ACE inhibitors, thus the half-life of bradykinin is prolonged. The levels of bradykinin generated in platelet concentrates filtered with negatively charged filters are variable (Shiba 1997). If the level is sufficiently high, 0.5-1 µg/kg body weight (Bonner 1990), it can cause hypotensive reactions. The interaction between negatively charged filters, bradykinin generation and ACE inhibitors is summarised in Figure 1.12.

The activation of the contact system may also lead to thrombin generation which can activate platelets.

Figure 1.12 The interaction between negatively charged filter, bradykinin generation and ACE inhibitors.

Dashed arrow shows the inhibitory effect of ACE inhibitors on kininase.



iv. Interaction with complement

The artificial surface of leucocyte removal filters can either activate complement via the alternative pathway or remove anaphylatoxins, C3a, C4a and C5a, depending on the type of filters used. Negatively charged polyester filters remove anaphylatoxins, which are positively charged, most likely by electrostatic interactions, whereas positively charged polyester filters increase the levels of anaphylatoxins (Snyder 1996, Geiger 1997).

1.4 PLATELET STORAGE LESION

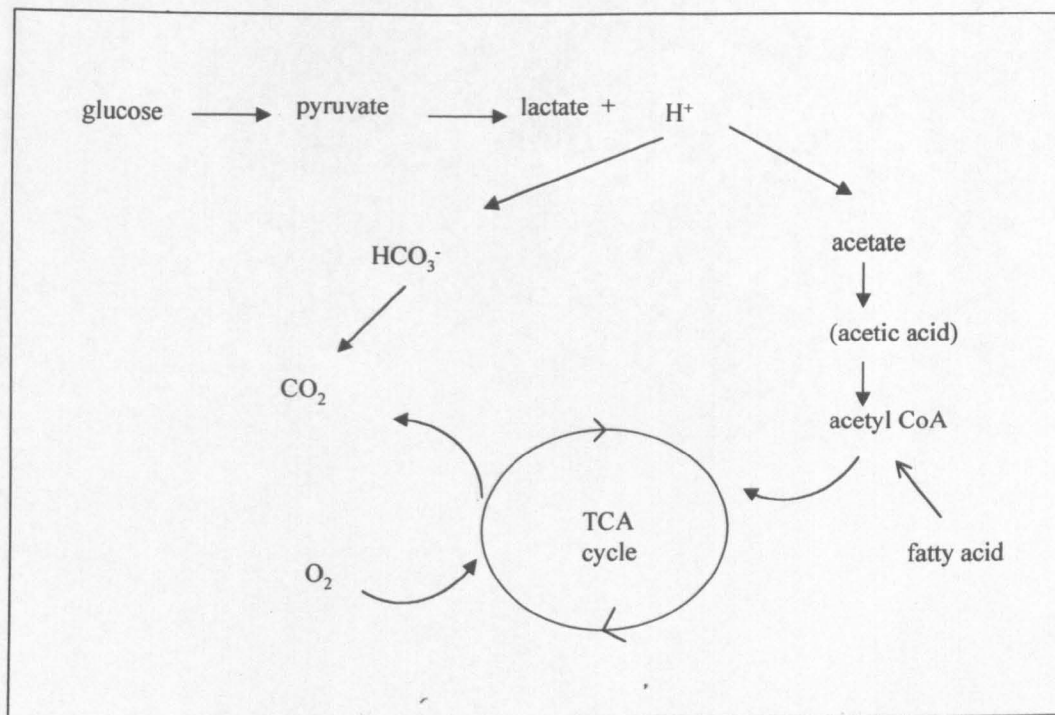
Despite the continual improvement in platelet storage conditions, deleterious changes in platelets during storage, the so called platelet storage lesion, resulting in impaired platelet function in vivo remains a challenging area to be resolved. Platelets are extremely sensitive to their microenvironment and the storage lesion starts from the

moment of blood collection from the donor to the administration to a patient. The platelet storage lesion encompasses multiple changes in platelet morphology, metabolism, activation and microvesiculation, leading to impaired functionality. Although much emphasis has been placed on the effect of pH on platelet storage lesion, several other factors during processing and storage of platelet concentrates can also influence the storage lesion. These include preparation method, composition of the storage bag, storage condition, and leucocyte content as described in section 1.5.

1.4.1 Platelet metabolism during storage

There are some differences between platelet metabolism in vivo and during in vitro. Although glucose is a significant substrate for oxidative phosphorylation of platelets in vivo, during the storage of platelet concentrates the major substrate appears to be plasma free fatty acid (Cesar 1987). Almost all glucose used is metabolised via glycolysis by converting through pyruvate to lactate and hydrogen ions. Hydrogen ions are buffered by bicarbonate in plasma and converted to water and carbon dioxide, which can leave through the wall of the storage container. There is only enough bicarbonate in plasma to buffer a rise in lactate concentration to approximately 20 mmol/L. Beyond that concentration, the pH will fall rapidly to below 6.3 where platelet viability will be lost (Murphy 1985, 1999). Very little pyruvate is decarboxylated to acetyl coenzyme A (acetyl CoA) and carbon dioxide. Instead acetyl CoA for entry into the tricarboxylic acid is derived from free fatty acid. Acetate used in some platelet storage media described below can also be converted to acetyl CoA. Figure 1.13 summarises the platelet metabolism in the storage conditions used for platelet concentrates for transfusion.

Figure 1.13 Platelet metabolism during storage.



1.4.2 Changes in platelet morphology

Platelet morphology remains the best practical indicator of the survival of stored platelets (Murphy 1994, Holme 1997). Even freshly prepared platelets appear to form some pseudopods (White 1992). Figures 1.14 and 1.15 show scanning electron micrographs of a discoid shape platelet as compared to a platelet with pseudopod formation. During storage a decline in pH, caused by lactic acid produced by glycolysis, as well as platelet activation, triggers the disc-to-sphere shape change, pseudopod formation, fusion of granule membranes to the open canalicular system, swelling of the open canalicular system, vacuolisation due to the release of granule contents, platelet aggregation, and microvesicle formation (White 1992, Klinger 1994, 1996).

1.4.3 Platelet activation

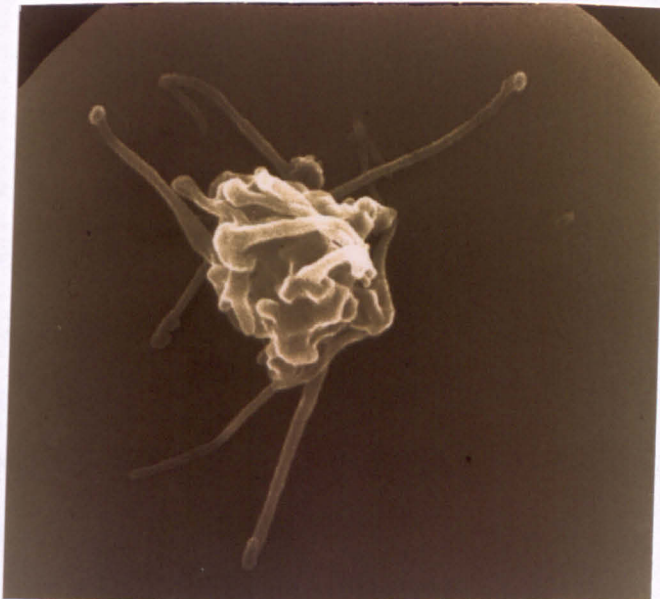
Trace amounts of thrombin and plasmin generated during blood collection and

contact of platelet membrane with the artificial surface of a storage container are reported to activate platelets (Michelson 1990). Artificial surfaces can activate platelet directly through the activation of specific ligands on the platelet membrane or indirectly through the generation of activated complements via the alternative pathway. The activation of platelets leads to a decrease of GPIb, increase of GPIIb/IIIa, P-selectin and CD63 expression on platelet membrane (Bode 1991, Rinder 1993). It is controversial whether the loss of GPIb is due to the proteolytic cleavage or resequestration into the canalicular system (Michelson 1990, Bode 1991, Divers 1995). The increase in GPIIb/IIIa expression is caused by the release of this glycoprotein from the storage pool inside platelets i.e. α -granules (George 1988, 1992).

Figure 1.14 A scanning electron micrograph of a discoid platelet. (x20,000)



Figure 1.15 A scanning electron micrograph of an activated platelet with pseudopod formation. (x20,000)



Although the same complex events (shape change, adhesion, release reaction and aggregation) which occur during the platelet response to various platelet agonists also take place in platelets during storage, they are not identical. Firstly, the above changes of stored platelets occur over several days as the result of continuous mild stimulation, in which the end products of platelet metabolism i.e. carbon dioxide and lactic acid may contribute to the degree of change. By contrast the activation of fresh platelets under laboratory conditions occurs and finishes in minutes. Secondly, platelets in stored platelet concentrates, which are unable to aggregate upon stimulation with low concentrations of ADP, still aggregate after incubation in fresh plasma (Rao 1992). Finally, platelets in stored platelet concentrates which express high level of P-selectin on the membrane, have reasonable recovery and survival in vivo (Michelson 1996). In contrast activated fresh platelets, which have passed the stage of reversible aggregation, are committed to complete the release reaction leading to the demise of the cells.

While a fair correlation between P-selectin expression and in vivo survival has been reported (Holme 1997), and some studies point to P-selectin expression as a signal for the clearance of activated platelets (Larsen 1989, Rinder 1991), some studies have provided evidence that increased P-selectin levels may not be an indicator of decreased in vivo circulation time (Michelson 1996, Berger 1998). Studies performed in baboons have shown that transfused activated platelets have similar recovery values to transfused resting platelets (Michelson 1996). It has been suggested that activated platelets shed their surface expressed P-selectin upon transfusion and remain in circulation as P-selectin-negative platelets. These degranulated platelets also continue to function in vivo as seen by their participation in bleeding time wounds, binding to Dacron in an arteriovenous shunt, and the generation of microvesicles (Michelson 1996).

1.4.4 Senescence of platelets

During storage, a fraction of platelets will reach the end of their normal life span and subsequently release the contents of their granule contents independently of activation-induced release reaction. Some released substances such as ADP activate the remaining platelets, whereas AMP reduces platelet reactivity (Fijnheer 1992).

The formation of platelet-derived microvesicles, another important phenomenon during in stored platelet concentrates, is described in detail in section 1.5.

1.5 PLATELET-DERIVED MICROVESICLES

1.5.1 Definition

There is no consensus on the precise definition of a platelet-derived microvesicle (Plt-MV). Nevertheless it is generally agreed that Plt-MV are particulate material derived from the membrane of intact platelets. Several definitions have been used to describe Plt-MV depending on the techniques employed. Using electron microscopy, Plt-MV are defined as membranous structures with highly polydispersed size and intracytoplasmic contents. They appear to be in the range of 1 μm or smaller, some contain cytoplasmic organelles and some are empty membranous sacs (Warren 1972). For assessment of their pro/anticoagulant properties, Plt-MV are defined as material isolated from platelet poor plasma by either ultracentrifugation or ultrafiltration (Wolf 1967). More recently flow cytometry has become a method of choice for the quantitative measurement of Plt-MV. In this technique, Plt-MV are defined by an arbitrary gate set by using 0.5, 1 or 2 μ latex particles or fluorescent beads or by using fresh or washed or gel filtered platelets (Miller 1987, Fox 1991, Pasquet 1996).

Both "microvesicle" and "microparticle" have been widely used in literature as synonyms. However, some consider the word "particle" is unspecific and undefined,

whereas "vesicle", as used in cell biology and pathology, has a more specific morphological meaning implying materials derived from endocytosis/exocytosis processes and containing cytoplasmic contents of the parent cell. There is ample evidence that Plt-MV generated by various mechanisms have different morphological and biological properties. In this thesis platelet-derived "microvesicle" is defined as material derived from the platelet membrane, regardless of its cytoplasmic contents and mechanism of generation.

Although this thesis mainly deals with MV derived from platelets (MV will use as an abbreviation for platelet derived microvesicles, unless otherwise stated), it should be noted that membrane microvesiculation is not unique to platelets. Red blood cell and leucocytes as well as some other eukaryotic cells can also undergo microvesiculation under various conditions although the mechanisms involved are not well defined. For example red cell-derived MV have been demonstrated during storage of red cell concentrates (Greenwalt 1991) and the increase in intracellular Ca^{2+} (Bucki 1998), monocyte-derived MV after stimulation by lipopolysaccharide (Satta 1994), lymphocyte-derived MV in human immunodeficiency virus (HIV) type 1 infection or after stimulation (Aupeix 1997, Armstrong 1998), endothelial cells after stimulation by thrombin (Bizios 1988), and MV derived from mouse sublingual glands during secretion (Bos-Vreugdenhil 1985).

1.5.2 Mechanisms of platelet-derived microvesicle formation

Several potential mechanisms of MV formation have been proposed with considerable evidence that the increase in cytoplasmic calcium is a key event in most scenarios. No single mechanism can explain the formation of MV in all cases and it is highly likely that the majority of mechanisms are interdependent as shown in Figure 1.16. Some proposed mechanisms are also described below.

i. Exocytosis

Using electron microscopy, this proposed mechanism of MV formation is based on the concept that upon activation MV are pre-formed inside platelets and released into the surrounding media when platelet membrane ruptures (Warren 1972). It is possible, however, that this finding was an artefact from the fixation process used in electron microscopy.

ii. Shear stress and mechanical injury

Microvesicles can be generated by blebbing of cell membranes under high shear stress. This mechanism is supported by the evidence that blood collected by intraoperative and postoperative salvage systems during cardiopulmonary bypass contains high levels of MVs (Sloand 1995).

iii. Destabilisation of membrane cytoskeleton

Different methods of physico-chemical disruption of the membrane cytoskeleton have shown to induce microvesiculation as shown in Figure 1.17. These include prolonged exposure to cold (4°C) or heat (49°C), freeze-thawing, alkali or acid pH and changes in calcium content (Bode 1994, George 1986). Upon exposure to cold, the microtubular ring disintegrates and bundling of actin filaments occurs, resulting in spheric transformation, pseudopod formation, and microvesiculation of platelets. The effect of pH on platelet activation and microvesiculation are shown in Figure 1.18. At a neutral pH most platelets are in discoid shape. At low and high pH platelets become activated indicating by discoid to sphere transformation and pseudopod formation leading to microvesiculation (Solberg 1986).

iv. Transbilayer movement of phospholipids

Upon activation by platelet agonists, an increase in cytoplasmic calcium occurs via either the transport of calcium from the external medium across the membrane or via the

release from the internal storage pools. The rise in cytoplasmic calcium triggers two potential mechanisms resulting in MV formation. Firstly, calcium induces the rearrangement of cytoskeletal proteins with protrusion and fusion of the apposing segments of plasma and/or granule membrane. This results in shedding of MV and transient formation of nonbilayer structures at the point where the membranes fuse to form MV. Secondly, the rise in cytoplasmic calcium produces a bi-directional movement of membrane phospholipids, the so called "flip-flop", with the outward transport of aminophospholipids occurring faster than inward transport of phosphatidyl choline, resulting in PS exposure and eversion of membrane membranes (Comfurius 1990, Bevers 1991).

v. Activation of calpain

Calpain is an endogenous calcium-dependent protease which is activated upon stimulation by physiological platelet agonists such as collagen and thrombin. Activated calpain generates MV by hydrolysis of actin-binding protein on the membrane. The role of calpain in MV formation is supported by the finding that the degree of calpain activation correlates positively with the amount of MV formation; and calpeptin, a membrane-penetrating calpain inhibitor, at concentrations that are sufficient to inhibit calpain-induced hydrolysis of actin-binding protein, inhibits the shedding of MVs (Fox 1991, Pasquet J-M 1996).

vi. Complement activation

The component which plays a major role in platelet activation and microvesiculation is the membrane attack complex, C5b-9. The assembly of C5b-9 on the platelet membrane leads to the influx of calcium from the extracellular medium across the plasma membrane and secretory-fusion of alpha-granules with the plasma membrane (Sims 1988).

vii. Antigen-antibody complexes

Antibodies which bind to the platelet membrane can act as a potent activator and generate MV through an undefined pathway. High levels of microvesicles are found in idiopathic thrombocytopenic purpura (ITP) (Jy 1992), and heparin-induced thrombocytopenia (Warkentin 1994). Moreover, purified IgG or sera from heparin-induced thrombocytopenic patients can stimulate normal platelets to generate MV (Warkentin 1994).

Figure 1.16 A schematic representation of the proposed mechanisms of microvesicle formation including exocytosis, transbilayer movement of phospholipids, activation of calpain and complement, and antigen-antibody reaction.

(From Krailadsiri P, Seghatchian J, Bode AP. Clin Appl Thromb/Hemost 1997;3:86-95.) ABP-actin binding protein, DB-dense bodies, DTS-dense tubular system, G-glycogen granules, TLC-translocase.

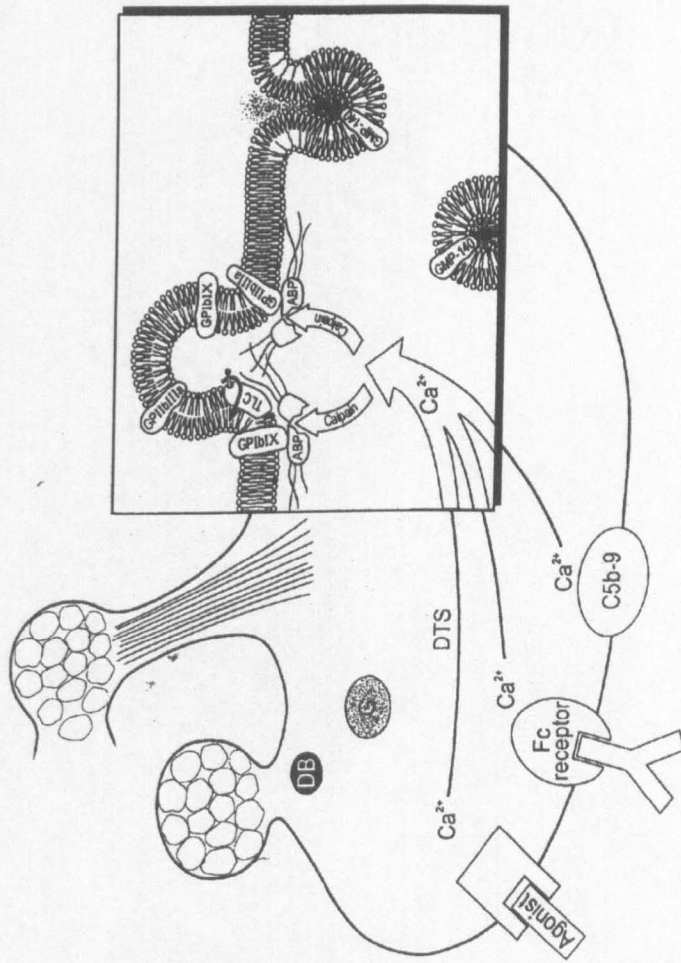


Figure 1.17 The changes in platelet cytoskeletal proteins and microvesicle formation induced by exposure to 4°C.

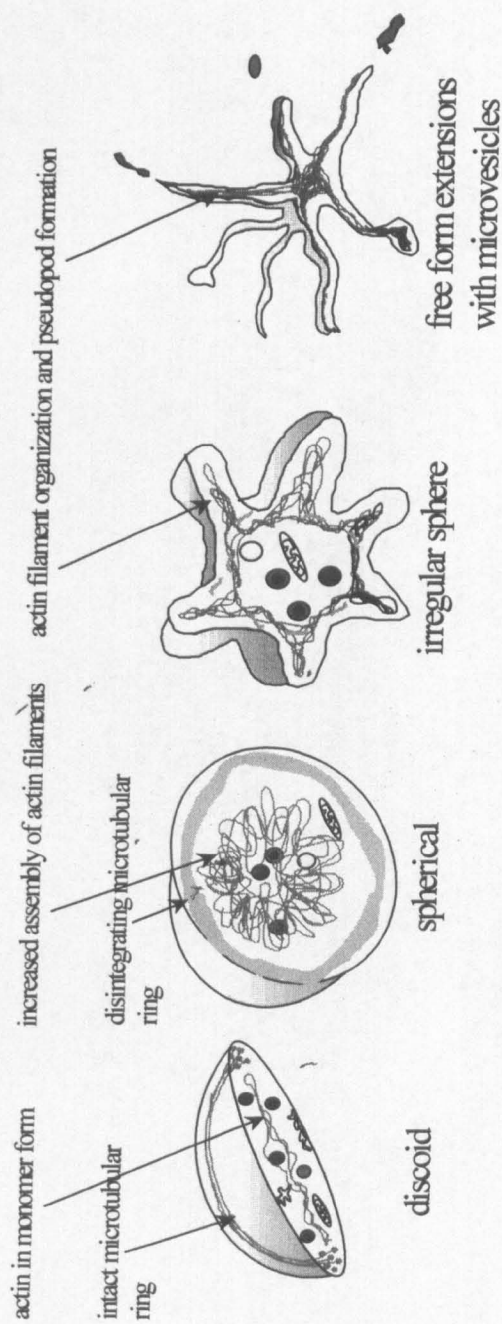
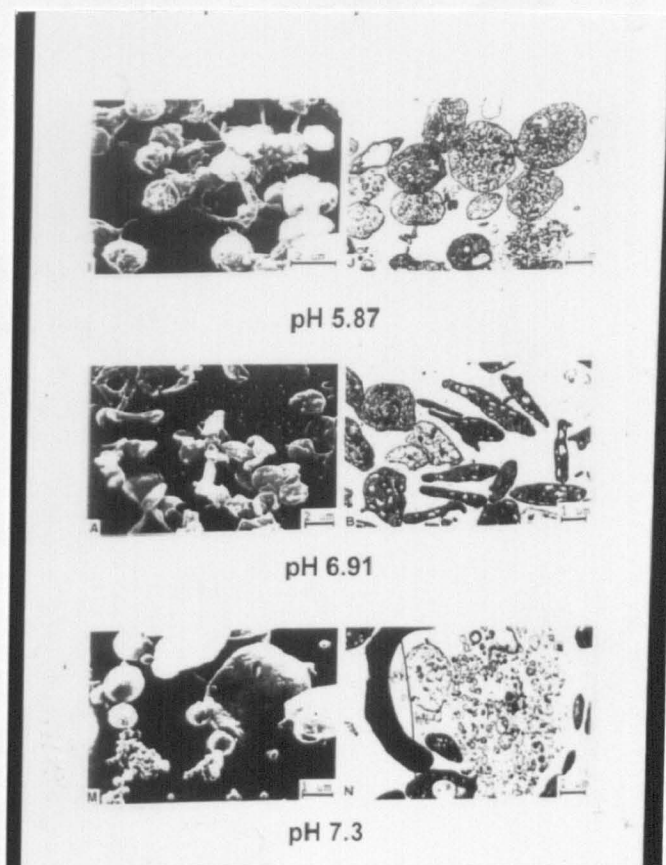


Figure 1.18 The effect of pH on platelet activation and microvesiculation.

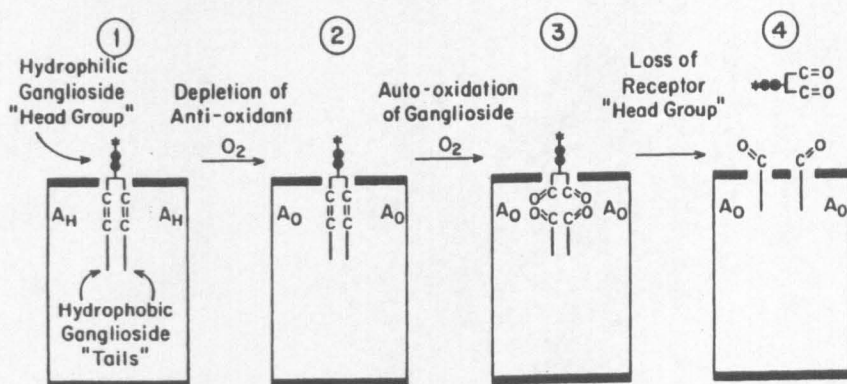
Left panels-scanning electron micrographs, right-transmission. At neutral pH, most platelets are discoid (middle panels). At low pH, platelets are activated and form pseudopods as well as microvesicles (top panels). At high pH, a high degree of microvesiculation occurs (bottom panels). (x4500)



viii. Peroxidation of platelet membrane lipid

It has been shown that all platelet membrane lipids are steadily lost during storage (Okuma 1971, Hamid 1980, Koerner 1986), while phospholipid membranes containing PF3 activity are increased in the supernatant plasma (Bode 1986, Solberg 1987). It is believed that this observation is related to platelet microvesiculation but it is unknown whether the loss of platelet membrane lipids is the cause or the effect. There are limited studies on platelet membrane lipids in stored platelet concentrates for transfusion. This is possibly because of the requirement of a special equipment and technology i.e. laser light scattering detection - high performance liquid chromatography. Nevertheless, it has been proposed that lipid peroxidation (auto-oxidation), a mechanism of lipid destruction in a system exposed to atmospheric oxygen, may be responsible for the loss of platelet membrane lipid (Koerner 1992). Figure 1.19 describes the proposed mechanism for peroxidation of platelet membrane lipid using ganglioside as an example. Other platelet membrane lipids can undergo peroxidation in the same fashion.

Figure 1.19 The proposed mechanism for peroxidation of platelet membrane lipid.



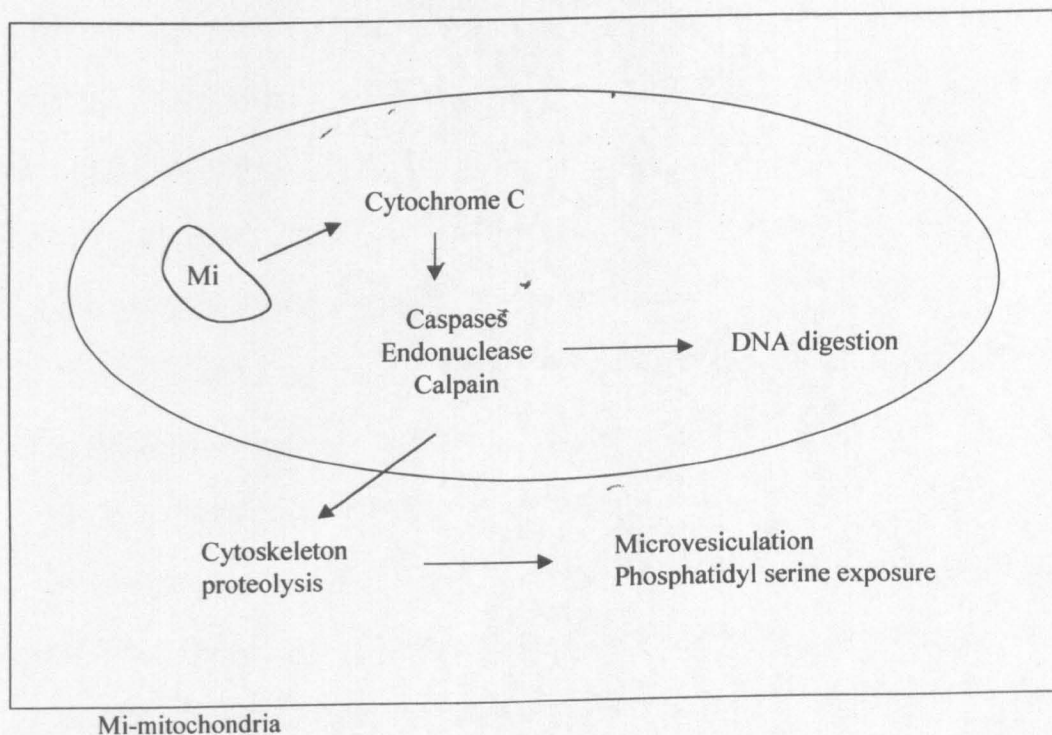
1. A lipid in the membrane is located next to an endogenous antioxidant (A_H) which is in its reduced form.
2. After oxygen has been continually diffused into the cell and all of the antioxidant has been converted to the oxidised form (A_O).
3. If more oxygen gets into the cell, a peroxidation of the unprotected lipid occurs, converting the olefinic double bond into two aldehydes and clipping the two hydrophobic lipid anchors that hold the lipid in the membrane.
4. The loss of polar head group, the moiety that is responsible for the specificity of lipid functional activity.

ix. Apoptosis

It has been suggested that microvesiculation is part of apoptosis or programmed cell death, an important physiological process in eukaryotic cells which allows the removal of damaged cells by phagocytosis in vivo (Steller 1995). Recently, it has been reported that platelets, despite being anucleated cells, undergo apoptosis in vitro (Vanags 1997, Li 1999). Some common features of apoptosis are illustrated in Figure 1.19.

Central to the apoptotic process is the activation of enzymes (caspases), which subsequently activate endonucleases and proteolytic enzymes through a cascade mechanism. The endonucleases digest DNA and the proteolytic enzymes digest proteins of the membrane cytoskeleton. The apoptotic cell disintegrates in an orderly manner into microvesicles, which are then cleared by phagocytosis *in vivo*. Cytochrome C release from mitochondria appears to be an important trigger by activating caspase, setting the cascade in motion. Exposure of phosphatidyl serine on the cell membrane surface has been shown in cells undergoing apoptosis (Steller 1995, Vanags 1997).

Figure 1.19 Some common features in apoptosis.



1.5.3 Microvesicle in platelet concentrates for transfusion

The presence of MV in platelet concentrates has long been demonstrated (Wolf 1967). However, limited studies on MV during the storage of platelet concentrates are available. Several processing methods currently used for the preparation of platelet

concentrates as well as sub-optimal storage conditions may influence the platelet storage lesion and the rate of MV formation as discussed below.

i. Collection and processing

Platelets are extremely sensitive to microenvironmental conditions. From the initial contact of blood with the collection tubing to the time of transfusion to the recipient, platelets are easily activated due to contact with artificial surfaces, mixing, centrifugation and cell-cell interaction. These can lead to MV formation. Different collection and processing techniques can influence platelet activation and microvesiculation to variable degrees. Platelets prepared from whole blood, where during the initial phase of collection platelets are exposed to a high concentration of anticoagulant, express a higher degree of activation and microvesiculation than those prepared from apheresis, where they are in contact with lower concentrations of anticoagulant (Sloand 1996). However, it should be noted that the different centrifugation g force used in both techniques could also account for the degree of platelet activation and microvesiculation in the final products.

More recently, leucocyte depletion has been implemented as a standard practice. Its potential effects on blood was discussed in section 1.3.

ii. Leucocyte content

Reports on the effect of residual leucocytes on platelet storage lesion are contradictory. While some studies have shown that leucocytes in platelet concentrates accelerate the platelet storage lesion (Sloand 1990), others have not found any difference between leucocyte depleted and non-leucocyte depleted products (Dzik 1992). The role of residual leucocytes in platelet microvesiculation is unknown but it is possible that proteases released from leucocytes such as elastase, cathepsin and other chymotrypsin-like enzymes, may contribute to proteolytic fragmentation/ microvesiculation of platelets.

iii. Storage containers and storage conditions

Several types of containers with different shape, size, thickness and plasticizer have been designed for platelet storage. However, the currently available containers are still not ideal in terms of blood-biomaterial compatibility characteristics and may contribute to platelet activation and MV formation. Complement activation via either the classical or alternative pathway occurs when blood is initially exposed to the collection tubing and container as well as during storage (Gyongyossy-Issa 1994). It is possible that the inner wall of most plastic containers provides a site for complement fixation that could interact with platelets. The observed benefit of a reduced container surface/volume ratio in terms of MV generation in certain experiments (Bode 1991) may be partially related to a reduction in platelet collision rate with active complement compounds on the container wall.

The pH of platelet concentrates has been considered as one of the major quality parameters. When pH falls below 6.1, platelets become spheric and form a large number of pseudopods on their surfaces which can lead to MV formation. High pH, above 7.3, induces platelet fragmentation, generating more MV than low pH (Solberg 1986).

The type of agitators used for the storage of platelet concentrates has been found to affect the degree of MV generation. Storage on an elliptical rotator leads to a higher concentration of MV compared with a flat bed agitator (George 1988).

1.5.4 Clinical Implications

Platelet-derived microvesicles are present in low levels in normal circulation. Higher levels have been reported in several pathological conditions in association with some clinical manifestations such as unstable angina (Singh 1995), diabetes mellitus (Strano 1991), sickle cell disease (Wun 1998), HIV infection (Holme 1998), as well as some other haematological disorders as described below. Although MV generated during processing and storage of blood components are infused along with the native cells, their

clinical significance have not been clarified. The possible clinical implications of MV in transfusion-related clinical scenarios are discussed below.

i. Haemostatic and thrombotic effects

Microvesicles contain high proportion of phosphatidyl serine on their surfaces and provide an optimal catalytic surface for tenase and prothrombinase complex formation, leading to significant thrombin generation *in vivo*. The procoagulant activity of MV could be accountable for the observation in ITP that the patient's tendency to bleed does not necessarily correlate with the degree of thrombocytopenia. Patients with higher concentrations of MV have fewer bleeding manifestations, e.g. fewer petechiae, less mucous membrane bleeding, than patients who have lower levels of MV (Jy 1992). High levels of MV associated with thrombotic risk were also found in paroxysmal nocturnal haemoglobinuria (Hugel 1999). Furthermore, a haemorrhagic tendency is also displayed in a rare disease, Scott syndrome, in which reduced shedding of MV upon platelet activation is observed (Weiss 1986).

It seems plausible that the procoagulant activity of MV may contribute to the therapeutic effects of cryoprecipitate or fresh frozen plasma on the correction of bleeding disorders in patients with uremia and platelet storage pool disease. Significant amounts of platelet contamination in plasma harvested to prepare fresh frozen plasma and cryoprecipitate leads to MV formation produced by freezing and thawing (George 1986). In cryoprecipitate, MV are concentrated more than 100 times that of the original plasma. Cryoprecipitate obtained from desmopressin (DDAVP)-treated donors, as routinely practiced in some countries, is reported to be much more effective than that from normal donors (Konecka 1990). The improvement in efficacy may be at least partly due to the high content of MV as DDAVP has been found to induce platelet microvesiculation (Horstman 1995).

Studies of frozen platelets have demonstrated their haemostatically effectiveness

to be superior to conventional liquid stored platelets, although the former shows shorter survival (Khuri 1999). This observation could be attributed to the MV, generated during the freeze-thawing process, which express higher level of bound factor V than intact platelets (Khuri 1999, Barnard 2000).

Infusible MV in freeze-dried form have been demonstrated to shorten bleeding time in thrombocytopenic patients although their circulatory half-life is markedly shorter than intact platelets (Chao 1996). Nevertheless, a multiple dose infusion of these MV may give a sustained haemostatic effect equivalent to stored PCs.

It should be noted that MV membrane expressing phosphatidyl serine provides a catalytic surface for anticoagulant as well as procoagulant reactions (Tans 1991, Dahlbäck 1992), although the evidence for the clinical importance of MV anticoagulant property has not been shown.

ii. Immunomodulation

It has been shown that the exposure of phosphatidyl serine on cell surfaces can trigger the reticuloendothelial recognition process leading to adherence of the cells to monocytes and macrophages and clearance from the circulation into liver and spleen (Zwaal 1996). This process is thought to be a significant mechanism of senescence for circulatory blood cells. It is possible that phosphatidyl serine on MV and activated platelets can also trigger monocyte/macrophage recognition and consequently accelerate the removal of MV as well as platelets from the circulation.

Microvesicles as well as activated platelets expressing P-selectin can adhere to neutrophils (Miyamoto 1998). This interaction is thought to correlate with the leucopenia seen in haemodialysis patients (Gawaz 1994). It remains to be elucidated whether this interaction leads to neutrophil activation or could potentially cause leucopenia leading to immunosuppression in recipients receiving blood components containing a large number of MV.

It has also been demonstrated that MV can activate adjacent platelets, endothelial, and monocytes, enhancing their adhesive interaction (Barry 1999a, b).

The exposed phosphatidyl serine on MV and activated platelet membranes can bind to certain plasma proteins such as prothrombin and β_2 -glycoprotein I, both of which have a high affinity to phosphatidyl serine and are relatively abundant in plasma. It has been proposed that this interaction may lead to the expression of neoantigens and providing that it is present long enough at a sufficiently high concentration in the circulation, it could cause antiphospholipid antibody formation (Comfurius 1995).

iii. Alloimmunisation

Microvesicles share several membrane antigens with intact platelets and it is possible that patients can be alloimmunised to these membrane antigens. This is supported by the evidence that some patients receiving cryoprecipitate develop platelet antibodies (George 1986). It seems likely that this is due to MV concentrated in the cryoprecipitate product as described previously.

iv. Transmission of infectious disease

More recently, attention has focussed on the hypothetical role of MVs in the transmission of variant Creutzfeldt Jacob disease by blood transfusion. This is related to the finding that platelets are the most abundant source of normal prion protein (PrP^c) in blood (MacGregor 1999, Prowse 1999) and upon activation by platelet agonists, PrP^c in platelets is expressed on the platelet surface in parallel to the platelet activation markers, CD62P (α -granule membrane protein) and CD63 (lysosomal membrane protein) (Holada 1999, Perini 1996). Furthermore, ruptured platelets containing CD62P also contain PrP^c . At least on a theoretical basis, MV could be carriers for abnormal prion protein as it is known to be membrane associated (Cashman 1990, Bendheim 1992).

1.6 WORK DESCRIBED IN THIS Ph.D. THESIS

The main objectives of this thesis are: i) to investigate whether leucocyte depletion generates or removes MV in platelet concentrates for transfusion, comparing between three in-process leucocyte removal filters for buffy coat derived platelets, and between various platelet preparation/leucodepletion methods; ii) to investigate whether various leucocyte filters and leucocyte depletion technologies activate the activation of coagulation/complement systems; iii) to investigate whether MV isolated from platelet concentrates can act as a catalytic surface for the coagulant and anticoagulant reactions; and iv) to apply soluble annexin V, an intracellular protein, to the assessment of platelet storage lesion.

Chapter 2 describes the general methods used in this study. For clarification, the methods of the assessment of MV procoagulant/anticoagulant properties are described separately in Chapter 7.

Chapter 3 investigates whether leucocyte depleted platelet concentrates prepared by the three techniques commonly used in the UK, i.e. filtration of buffy coat derived platelets, MCS+ LD and Cobe LRS apheresis, have the same functional reserve as measured by the changes in platelet size indices upon exposure to EDTA.

Chapter 4 investigates whether leucocyte removal filters for buffy coat derived platelets generate or remove MV, as well as whether different filters made from different synthetic materials, i.e. negatively charged polyester, positively charged polyester, and neutrally charged (non-charged) polyurethane, have the same effects.

Chapter 5 investigates whether the three commonly used platelet preparation/leucodepletion methods have the same effects on the generation and removal of MV.

Chapter 6 investigates whether various filters as well as various leucodepletion technologies have the same effects on the activation of coagulation and complement

systems

Chapter 7 investigates whether MV isolated from platelet concentrates can act as a catalytic surface for the coagulant and anticoagulant reactions.

Chapter 8 investigates whether soluble annexin V can be used to assess platelet storage lesion.

Finally, general discussion and references are presented in Chapters 9 and 10, respectively.

MATERIALS AND METHODS**2.1 FULL BLOOD COUNTS**

For platelet enumeration, an automated haematology analyser based on the direct current (DC) principle, Sysmex SE-9000 (TOA Medical Electronics Co. Ltd, Kobe, Japan), was used. After the blood sample is aspirated at a predetermined volume and diluted, it is sent to each detection chamber containing a small opening called aperture. On each side of the aperture, there is an electrode through which DC flows. When the blood cells pass through the aperture in a single file, the DC resistance between the electrodes changes, causing changes in the electrical pulse proportional to the size of the blood cell. Data on electrical pulses are collected and used to draw a particle size distribution which reflects the size of the blood cells.

Platelets are measured in the same detection chamber as red cells using lower and upper discriminators (LD and UD) set automatically for each type of cell. The red blood cell count is obtained from the number of particles with size between 25-75 fL (LD) and 200-250 fL (UD). The platelet count is obtained from the number of particles with size between 2-6 fL (LD) and 12-30 fL (UD).

2.2 FLOW CYTOMETRY TECHNIQUES

A Coulter Epics XL flow cytometer (Coulter Electronics, Miami, Florida, USA) was used. The instrument was calibrated for fluorescence and light scattering using the manufacturer's standard beads, Flow-Check fluorospheres (Coulter Electronics). FL1

(fluorescein isothiocyanide, FITC), FL2 (R-phycoerythrin, RPE), FL3 (phycoerythrin-Texas red), and FL4 (phycoerythrin-cyanide 5, PC5) were detected using 525, 575, 620, and 675 nm band pass filters, respectively. All data were obtained with gain settings in the logarithmic mode.

The optimal volume of each fluorochrome-labelled monoclonal antibody was determined by a titration curve. To ensure that the antibodies were always present in excess, twice the optimal volume was used. All antibodies were used undiluted. Each new batch of antibodies was titrated against the previous one.

Flow-Check fluorospheres (Coulter Electronics, Miami, Florida, USA) were used for daily quality control. Following the manufacturer's instruction, the target range for each parameter was determined by collecting 20 data points over at least 5 days, using the same instrument settings as in the test protocols, and the mean of peak intensity \pm 2 standard deviations was calculated. The peak intensities of FS (forward scatter signal), SS (side scatter signal), FL1, FL2, FL3, and FL4 were monitored and if necessary the voltage and amplification were adjusted so that the values fell within the target ranges.

2.2.1 Absolute leucocyte count in leucocyte depleted blood components

i. Principle

The levels of leucocytes (WBC) in platelet concentrates are below the lower detection limit of automated cell counters, i.e. less than $0.5 \times 10^9/L$, and therefore require a more sensitive method of measurement such as flow cytometry. The WBC are stained with propidium iodide (PI), which is a nucleic acid dye and when used with ribonuclease (RNase), stains only DNA. Platelets and red cells do not contain deoxyribonucleic acid (DNA), and their ribonucleic acid (RNA) is digested by RNase, so that they do not stain with PI. The reagent also contains detergent which permeabilises cell membranes to allow the entry of PI. The absolute WBC counts are obtained by using a known number of fluorescent beads as an internal reference.

ii. Reagents

All reagents were part of the LeucoCOUNT kit (Becton Dickinson, San Jose, California, USA).

1. LeucoCOUNT reagent containing propidium iodide, RNase and detergent
2. TruCOUNT tube containing a known number (shown on the package) of 4.2 µm lyophilised fluorescent beads

iii. Method

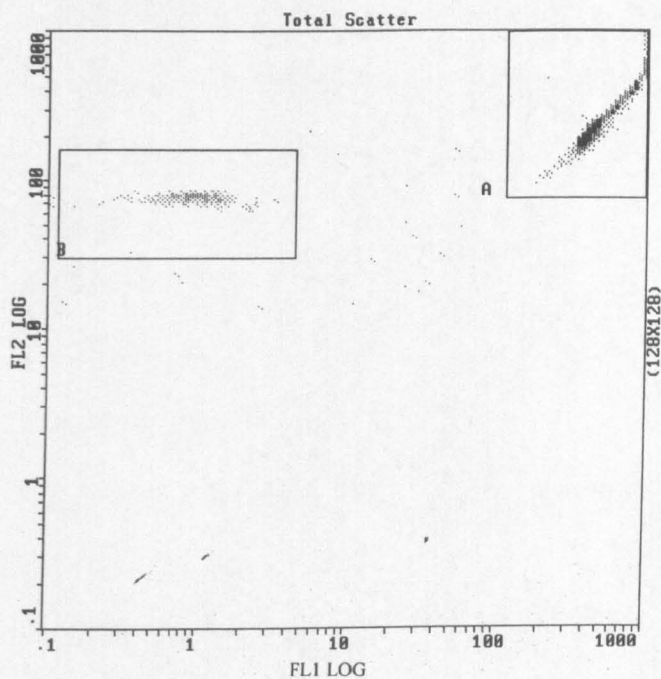
1. Pipette 100 µL of sample into a TruCount tube.
2. Add 400 µL of LeucoCOUNT reagents
3. Vortex gently for 10 seconds.
4. Incubate the tube for five minutes at room temperature in the dark.
5. Analyse samples within 1 hour.
6. Acquire 10000 events of fluorescent beads. A flow cytometric plot of leucocytes and beads is shown on Figure 2.1.
7. Calculate the absolute leucocyte count by using the following formula:

$$\text{Absolute WBC}/\mu\text{L} = \frac{\text{No. of beads per TruCOUNT tube} \times \text{WBC events (B)}}{\text{Bead events (A)} \times \text{Volume of sample stained } (\mu\text{L})}$$

Figure 2.1 Absolute WBC count using flow cytometry.

Region A-fluorescent bead, region B-WBC.

$$\text{Absolute WBC}/\mu\text{L} = \frac{\text{No.of beads per TruCOUNT tube} \times \text{WBC events (B)}}{\text{Bead events (A)} \times \text{Volume of sample stained } (\mu\text{L})}$$



2.2.2 Expression of P-selectin (CD62P, GMP-140) on the platelet surface membrane

i. Principle

P-selectin is an α -granule membrane protein. Upon platelet activation the α -granule membrane fuses with the platelet membrane and P-selectin is expressed on the platelet surface, where it can be detected by a fluorescein-conjugated monoclonal antibody specific to P-selectin.

ii. Reagents

1. Fluorescein-conjugated mouse IgG1 monoclonal antibody specific to human P-selectin (Serotec, Oxford, UK)
2. Fluorescein-conjugated mouse IgG1 Isotype control antibody (Serotec, Oxford, UK)
3. Phosphate-buffered saline (PBS) (Sigma-Aldrich Company Ltd, Dorset, UK)
4. 0.5% Formaldehyde (Sigma-Aldrich Company Ltd, Dorset, UK) in PBS
5. 12x75 mm Polystyrene tubes (Falcon 2052, Becton Dickinson Labware, Lincoln Park, New Jersey, USA)

ii. Method

1. In the first tube add 5 μ L anti-CD62P, in the second tube add 5 μ L of an isotype control antibody
2. Add 50 μ L PBS to each tube.
3. Add 2 μ L platelet concentrate sample to each tube.
4. Incubate at room temperature in the dark for 20 minutes.
5. Add 1 mL 0.5% formaldehyde in PBS.
6. Analyse the sample on flow cytometer within one hour. For each sample a

region was set to include 1% of total events in the positive region using an isotype control (Schmitz 1998).

2.2.3 Microvesicle count

i. Principle

Microvesicles express the same membrane antigens as their parent cells, i.e. platelet membrane glycoproteins IIb/IIIa (CD41/CD61) for platelet derived (Plt-MV), glycophorin A for red blood cell derived (RBC-MV), and CD45 for white blood cell derived microvesicles (WBC-MV). They can thus be detected by flow cytometry using fluorochrome labelled monoclonal antibodies specific to CD41, glycophorin A, and CD45, respectively. MV are differentiated from the instrument background by their fluorescent signals and from the native intact cells by forward scatter signal (FS), which reflects the size of the particle. Using microvesicle containing plasma, the absolute count of MV was obtained by adding a known number of fluorescent beads as a reference (Miller 1987, Combes 1997).

ii. Reagents

1. Mouse IgG1 anti-human CD41-RPE, mouse IgG1 anti-human glycophorin A-FITC (Dako Ltd, Cambridge, UK), and mouse IgG1 anti-CD45-PC5 (Coulter Electronics, Miami, Florida, USA)
2. TruCount tube (Becton Dickinson, San Jose, California, USA) containing a known number of lyophilised fluorescent beads
3. PBS pH 7.4 (Sigma-Aldrich Company Ltd, Dorset, UK) filtered through a 0.2 μ filter twice and degassed
4. 0.5% paraformaldehyde in PBS (Sigma-Aldrich Company Ltd, Dorset, UK)

iii. Method

1. For Plt-MV, centrifuge 1 mL of platelet concentrates in a 1.5

polypropylene eppendorf tube (BDH-Merck, Leicester, UK) at 16100 g for 1 minute.

2. Transfer 300 μL of supernatant plasma into another tube.
3. Using a TruCount tube, add 5 μL anti CD41-RPE, 5 μL of anti glycophorin A-FITC, 50 μL supernatant plasma and 100 μL PBS.
4. Incubate at room temperature in the dark for 20 minutes.
5. Add 1 mL of 0.5% paraformaldehyde in PBS.
6. Analyse the sample immediately, before analysis vortex the tube at 1400 rpm for 5 seconds.
7. Acquire 2000 beads with minimal threshold (1) on side scatter (SS).
8. Calculate the number of MV using the following formula:

$$\text{MV count (x10}^3\text{/mL)} = \frac{\text{total number of beads x number of MV counted}}{\text{number of beads counted x volume of sample (}\mu\text{L)}}$$

iv. MV region setting

The region setting for Plt-MV is arbitrary as their size and intracellular contents are heterogeneous, and there is no clear demarcation between Plt-MV and intact platelets. However, once the regions for MV were set as described below, they were kept constant throughout the whole period of study. This, together with the daily quality control of the flow cytometer, enabled comparative analysis between results obtained from different runs.

The region for Plt-MV was set by using gel filtered fresh platelets. Using a Sepharose 2B gel column (Pharmacia, Piscataway, New Jersey, USA), intact platelets having bigger size eluted in the early fractions, where as Plt-MV having smaller size eluted in the later fractions. The early fractions containing almost all intact platelets was used to set the Plt-MV region by including 1% of the population with the lowest FS signal (Figure 2.2).

For RBC-MV the region was set by using day 35 red cell concentrates and all events with FS signal lower than the homogeneous intact RBC population were classified as RBC-MV (Figure 2.3).

For WBC-MV the region was set by using 0.2 mmol/L calcium (Ca) ionophore A23187 (Sigma-Aldrich Company Ltd, Dorset, UK) activated WBC separated by gradient media (Figure 2.4).

v. Assay linearity

Linearity of each assay was performed by serially diluting samples obtained from day 6 platelets or day 35 red cell concentrates in fresh AB plasma which had been double filtered through 0.2 μ filters. The linearity of Plt-MV and RBC-MV assays is shown in Figure 2.5. The lower detection limit of Plt-MV and RBC-MV was 50/ μ L (50×10^3 /mL). For WBC-MV, even with the use of enriched WBC (93×10^9 /L) activated by high concentration of Ca ionophore, only 36×10^3 /mL were generated. Although repeated assays gave a coefficient of variance of 10%, further dilutions to establish linearity did not provide accurate results.

During a pilot study, non-leucocyte depleted and leucocyte depleted platelet concentrates were used to validate MV assays. While Plt-MV and RBC-MV were detected at all times, WBC-MV were not. This was most likely because the concentration of intact WBC (approximately 100/ μ L and less than 20/ μ L in non-leucocyte depleted and leucocyte depleted platelets, respectively) was much lower than platelets and RBC, and thus the level of WBC-MV was lower than the detection limit.

Figure 2.2 Region setting for Plt-MV.

Top-gel filtered platelets, bottom-day 6 platelets. Using gel filtered platelets, Plt-MV region was set by including 1% of the population with the lowest FS signal.

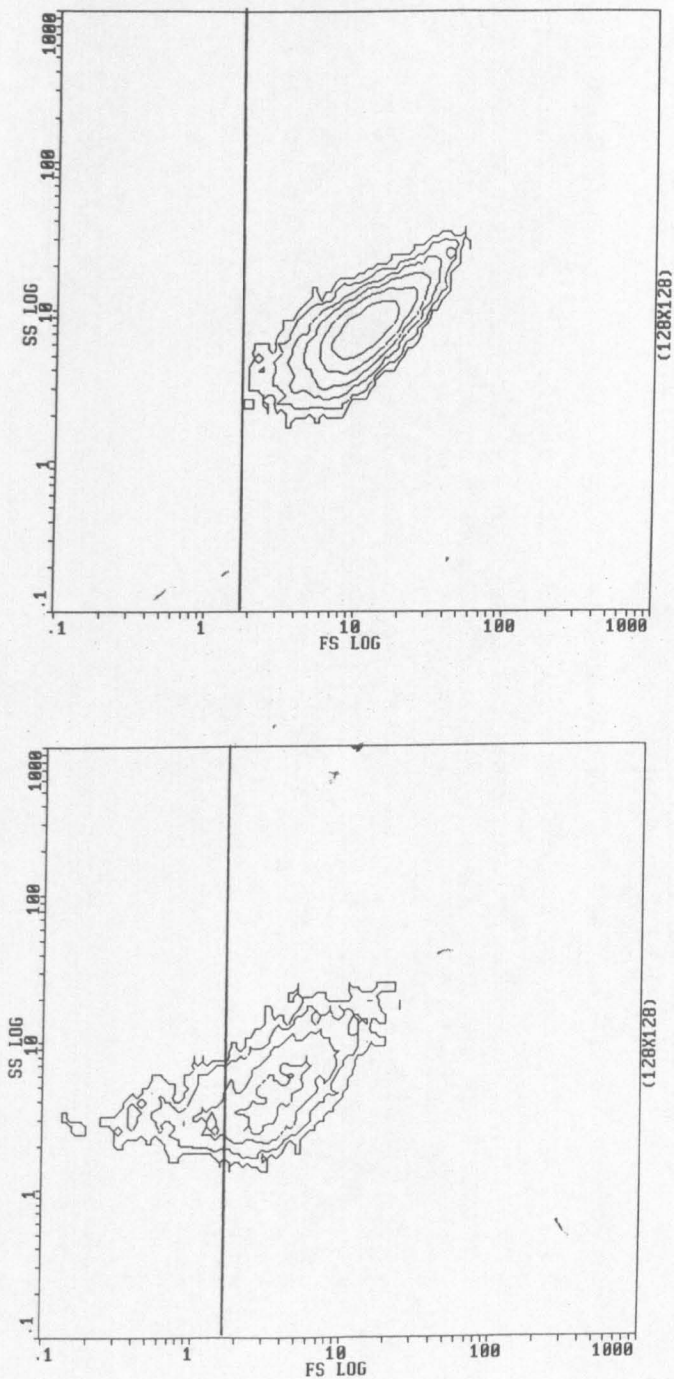


Figure 2.3 Region setting for RBC-MV.

Using day 35 red cell concentrates, a region of RBC-MV was set to include all events with FS signal lower than intact RBC.

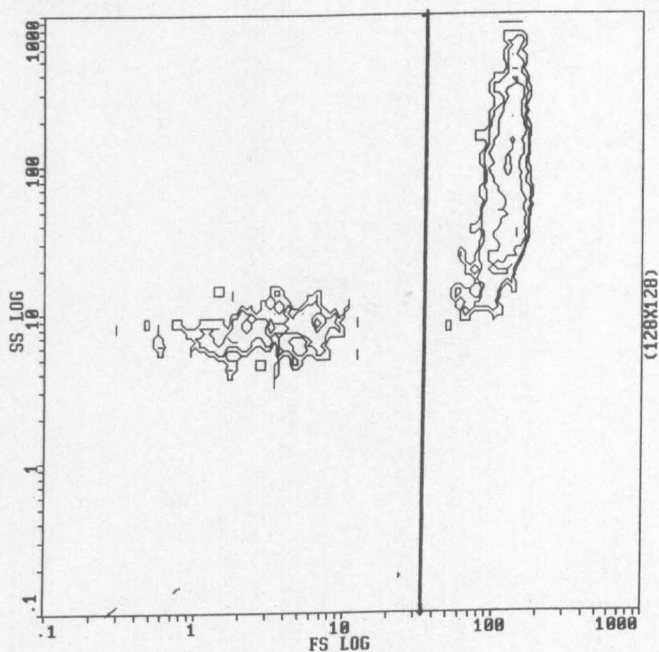
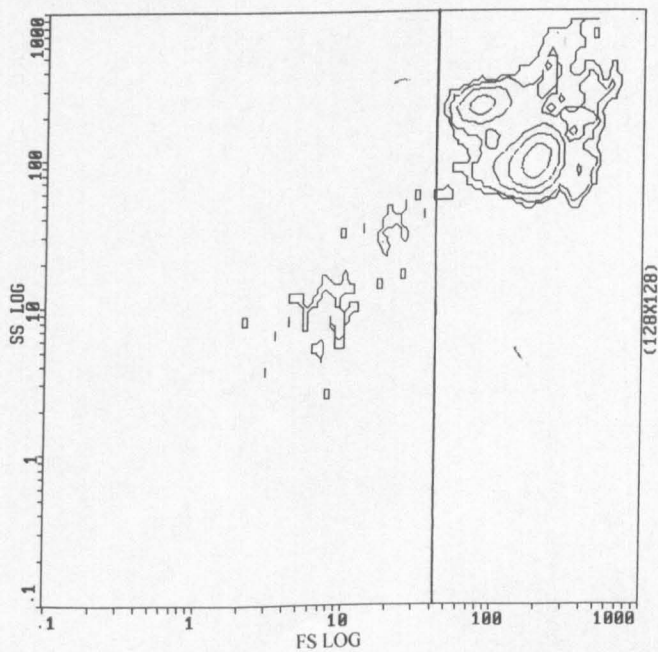


Figure 2.4 Region setting for WBC-MV.

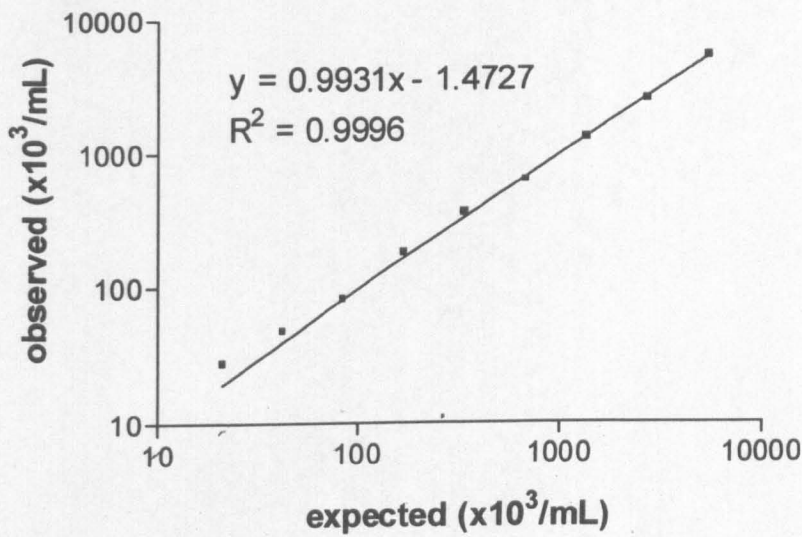
Using gradient media enriched, Ca ionophore activated WBC, the region for WBC-MV was set to include all events with FS signal lower than intact WBC.



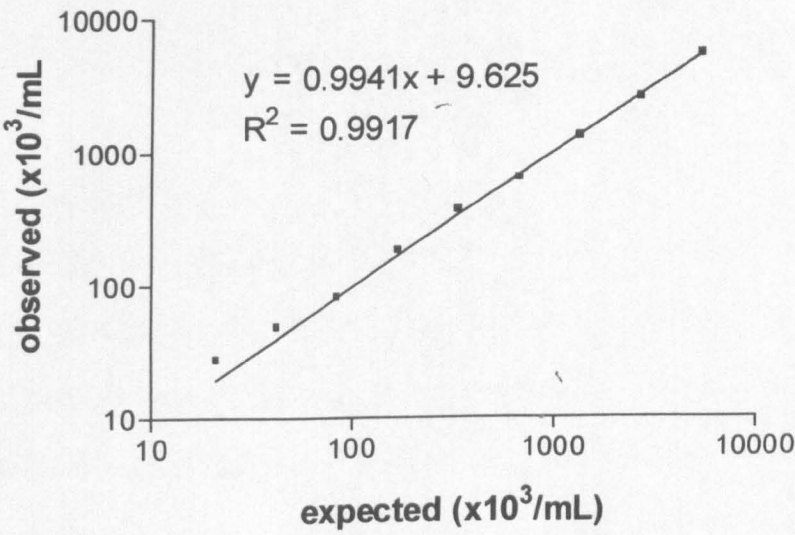
2.5 Linearity of microvesicle count.

Top-Plt-MV, middle-RBC-MV. _____ ideal line where $r^2=1$. Linearity of each assay was performed by serially diluting samples obtained from day 6 platelets or day 35 red cell concentrates in fresh AB plasma which had been double filtered through 0.2 μ filters.

Plt-MV



RBC-MV



2.3 PREPARATION OF PLATELET POOR PLASMA (PPP)

Platelet poor plasma was prepared by spinning 40 mL of platelet concentrate in a 50 mL sterilised graduated polypropylene centrifuge tube (BDH-Merck Ltd, Leicester, UK) at 3000 rpm (2000 g) at 22 °C for 30 minute with the brake off, using a bench top centrifuge (IEC Centra-7, International equipment company, Miami, Florida, USA). Thirty-eight mL supernatant were transferred to another tube before being aliquotted (0.5 mL) into 1.5 mL microtubes (Sarstedt, Numbrecht, Germany). Samples were kept frozen at -40°C for further testings. The PPP prepared by this method was used for various enzyme linked immunosorbent (ELISA) as well as chromogenic assays on kallikrein-like and thrombin-like activities.

2.4 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA were used to measure supernatant levels of markers of platelet activation (P-selectin), activation of coagulation system (FXIIa, prothrombin fragment 1+2, thrombin-antithrombin complex, complement activation (C3a) and platelet storage lesion (annexin V).

General principle

All the ELISA used employ a direct sandwich method. Plastic microtitre plates are coated with an antibody specific to the protein (antigen) of interest by passive adsorption (capture antibody). Unbound antibody is removed by washing the plate. Test sample containing antigen of interest is added and incubated. The unbound antigen is removed by washing the plate. A second antibody (detection antibody) against the antigen of interest but not for the same epitope(s) as the capture antibody, conjugated to an enzyme, peroxidase or alkaline phosphatase, is added. The reaction is visualised by

the addition of a colourless substrate, which is then cleaved by the enzyme conjugate to produce a coloured product. The colour produced is measured by spectrophotometry and is proportional to the amount of antigen bound to the plate.

Quality control

A quality control plasma was included in all plates using the control plasma provided in ELISA kits. An in-house reference plasma was also included to monitor inter-assay variation. The reference plasma was prepared by pooling platelet poor plasma obtained from 5 units of day 3 platelet concentrates. The acceptable range for this plasma was defined as the mean \pm 2 standard deviations from assays performed on 10 separate occasions. Results from assays where either the control plasma fell outside the range defined by manufacturer or the reference plasma fell outside the acceptable range were rejected. All assays were performed in duplicate. If the CV between duplicates was greater than 10%, the results were rejected.

2.4.1 Soluble P-selectin (sP-selectin)

i. Principle

The P-selectin expressed on platelet surface membrane upon platelet activation can be cleaved into the plasma, so called soluble P-selectin (sP-selectin).

ii. Reagents

All reagents were part of a kit from R&D System (R&D Systems Europe Ltd., Oxford, UK).

1. Microtitre plate coated with a murine monoclonal antibody to human sP-selectin
2. Sheep polyclonal antibody to recombinant human sP-selectin conjugated to horseradish peroxidase - diluted according to manufacturer's instruction
3. sP-selectin standards consisting of 6 concentrations of sP-selectin ranging from 0-50 ng/mL, reconstituted according to the manufacturer's

recommendation

4. sP-selectin control - reconstituted according to the manufacturer's recommendation
5. Substrate - tetramethylbenzidine
6. Sample diluent
7. Conjugate diluent
8. Wash buffer concentrate - diluted according to manufacturer's instruction
9. Stop solution containing acid

iii. Method

1. Dilute control, test and reference plasma 1 in 20 with sample diluent.
2. Add 100 μL of standards, diluted control, reference and test plasma to each well.
3. Add 100 μL sP-selection conjugate to each well.
4. Incubate at room temperature for 1 hour.
5. Wash the plate 3 times by adding 300 μL of wash buffered solution per well.
6. Add 100 μL of substrate.
7. Add 100 μL stop solution.
8. Measure the absorbance at 450 nm with reference wavelength set at 620 nm.
9. The concentrations of P-selectin were derived from a linear-linear four parameter logistic plot standard curve. The control, test and reference results were multiplied by the dilution factor.

2.4.2 FXIIa

i. Principle

Factor XII is activated and becomes αFXIIa , upon exposure to negative charged

surfaces. α FXIIa binds to surfaces and is protected from plasma protease inhibitors. Further proteolytic cleavage of α FXIIa leads to the formation of β FXIIa which does not have the surface binding property and is inhibited by inhibitors such as C1 inhibitor. The assay used measured both α FXIIa and β FXIIa.

ii. Reagents

All reagents were part of a kit from Shield Diagnostics (Shield Diagnostics Ltd., Dundee, UK).

1. Microtitre plate coated with a mouse monoclonal antibody to activated FXII
2. Sheep polyclonal antibody to human activated FXII conjugated to alkaline phosphatase - diluted according to manufacturer's instruction
3. Activated FXII standards consisting of 5 concentrations of purified activated FXII ranging from 0-20 ng/mL, reconstituted according to the manufacturer's recommendation
4. Activated FXII controls - reconstituted according to the manufacturer's recommendation
5. Substrate - phenolphthalein monophosphate
6. Wash buffer concentrate - diluted according to manufacturer's instruction
7. Stop solution containing sodium hydroxide and EDTA

iii. Method

1. Add 100 μ L of standards, control, and test plasma to each well.
2. Incubate at room temperature for 1 hour.
3. Wash the plate 5 times by adding 300 μ L of wash buffered solution per well.
4. Add 100 μ L of conjugate.
5. Incubate at room temperature for 1 hour.

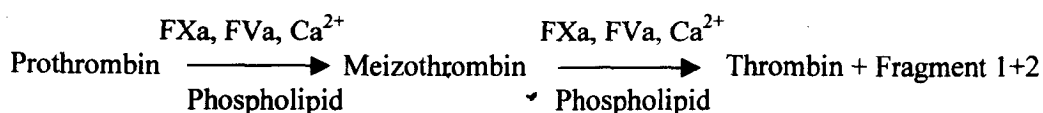
6. Wash the plate 5 times by adding 300 μL of wash buffered solution per well.
7. Add 100 μL substrate to each well.
8. Incubate at room temperature for 15 minutes.
9. Add 100 μL stop solution.
10. Measure the absorbance at 550 nm.
11. The concentrations of FXIIa were derived from a linear-linear four parameter logistic plot standard curve.

2.4.3 Prothrombin fragment 1+2 (F1+2)

i. Principle

The conversion of prothrombin to thrombin generates F1+2 as shown below.

Therefore the level of F1+2 can be used to quantify the amount of thrombin generated.



ii. Reagents

All reagents were part of a kit from Dade Behring (Dade Behring, Marburg, Germany)

1. Microtitre plate coated with rabbit antibodies to human F 1+2
2. Rabbit anti-human prothrombin conjugated to peroxidase
3. Conjugate buffer containing Tris, Tween, and bovine serum albumin
4. F 1+2 standards, concentrations range 0.04 to 10 nmol/L - diluted according to manufacturer's instruction
5. F 1+2 control plasma - diluted according to manufacturer's instruction
6. Sample buffer containing Tris, Tween and sodium chloride

7. Wash solution containing phosphate and Tween - diluted according to manufacturer's instruction
8. Substrate buffer containing hydrogen peroxide in citrate-buffer solution
9. Substrate - o-phenyldiamine-dihydrochloride
10. Stop solution - 0.5 N sulphuric acid

iii. Methods

1. Dilute standards, control, and test plasma 1 in 2 with sample buffer.
2. Add 100 μ L of diluted standards, control, and test plasma to each well.
3. Incubate at 37 °C for 30 minutes.
4. Wash the plate three times by adding 300 μ L of wash solution per well.
5. Add 100 μ L of conjugate into each well.
6. Incubate at 37 °C for 30 minutes.
7. Same as step 4.
8. Add 100 μ L substrate and incubate at room temperature in the dark for 15 minutes.
9. Add 100 μ L stop solution.
10. Measure the absorbance at 492 nm.
11. Plot a log-log point to point standard curve. Read the control and test results against the standard curve.

2.4.4 Thrombin-antithrombin III complex (TAT)

i. Principle

Once thrombin is generated, it forms a complex with a natural inhibitor in plasma, antithrombin III.

ii. Reagents

All reagents were part of a kit from Dade Behring (Dade Behring, Marburg, Germany)

1. Microtitre plate coated with rabbit antibodies to human thrombin
2. Rabbit anti-human ATIII conjugated to peroxidase
3. Conjugate buffer containing Tris buffer and bovine serum albumin
4. TAT standards, concentrations range 2 to 60 $\mu\text{g/L}$ - diluted according to manufacturer's instruction
5. TAT control plasma - diluted according to manufacturer's instruction
6. Sample buffer containing Tris, Tween and EDTA
7. Wash solution containing phosphate and Tween - diluted according to manufacturer's instruction
8. Substrate buffer containing hydrogen peroxide in citrate-buffer solution
9. Substrate - o-phenylenediamine-dihydrochloride
10. Stop solution - 0.5 N sulphuric acid.

iii. Methods

1. Dilute standards, control, and test plasma 1 in 2 with sample buffer.
2. Add 100 μL of standards, diluted control, and test plasma to each well.
3. Incubate at 37 °C for 15 minutes.
4. Wash the plate three times by adding 300 μL of wash solution per well.
5. Add 100 μL of conjugate into each well.
6. Incubate at 37 °C for 15 minutes.
7. Same as step 4.
8. Add 100 μL substrate and incubate at room temperature in the dark for 30 minutes.
9. Add 100 μL stop solution.
10. Measure the absorbance at 492 nm.
11. Plot a log-log linear regression standard curve. Read the control and test results against the standard curve.

2.4.5 Complement C3a

i. Principle

C3a is generated during the activation of complement system via the classical or the alternative pathway. C3a is short-lived and is cleaved into C3a-desArg by removal of an arginine residue. This assay measures C3a-desArg.

ii. Reagents

All reagents were part of a kit from Quidel (Quidel, San Diego, CA, USA).

1. Microtitre plate coated with mouse monoclonal anti-human C3a
2. Rabbit anti-C3a conjugated to peroxidase
3. C3a standard - diluted according to manufacturer's instruction
4. C3a control plasma - diluted according to manufacturer's instruction
5. Sample buffer - diluted according to manufacturer's instruction
6. Wash solution - diluted according to manufacturer's instruction
7. Substrate - tetramethylbenzidine
8. Stop solution - 1 N sulphuric acid

iii. Methods

1. Reconstitute and dilute standard and control according to manufacturer's instruction.
2. Dilute test plasma 1 in 100 with sample buffer.
3. Add 100 μ L of standards, diluted control, and test plasma to each well.
4. Incubate at room temperature for 1 hour.
5. Wash the plate three times by adding 300 μ L of wash solution per well.
6. Add 100 μ L of conjugate into each well.
7. Incubate at room temperature for 1 hour.
8. Same as step 4.
9. Add 100 μ L substrate and incubate at room temperature for 15 minutes.

10. Add 100 μ L stop solution.
11. Measure the absorbance at 450 nm.
12. Plot a linear-linear point to point standard curve. Read the control and test results against the standard curve.

2.4.6 Annexin V

i. Principle

Annexin V is an intracellular protein which is released upon cellular damage. The detailed property of annexin V is discussed in Chapter 9.

ii. Reagents

All reagents were part of a kit from Asserachrom (Stago, Genevilliers, France) unless otherwise stated.

1. Microtitre plate coated with rabbit polyclonal anti-human annexin V
2. Polyclonal anti-annexin V peroxidase conjugate - diluted according to manufacturer's instruction
3. 100 ng/mL annexin V standard - reconstituted according to manufacturer's instruction
4. Substrate - o-phenylenediamine dihydrochloride in distilled water containing urea peroxide
5. Dilution buffer
6. Wash solution
7. 3 M sulphuric acid

iii. Method

1. Dilute annexin V standard with dilution buffer to the concentrations of 20, 10, 5, 2, and 0.5 ng/mL.
2. Dilute reference and test plasma 1:5 with dilution buffer.
3. Add 200 μ L of standards or diluted plasma to each well and incubate at

room temperature for 2 hours.

4. Wash the plate 5 times by adding 300 μ L of washing solution per well.
5. Add 200 μ L of anti-annexin V peroxidase conjugate per well.
6. Incubate the plate at room temperature for 2 hours.
7. Wash the plate 5 times by adding 300 μ L of washing solution per well.
8. Add 200 μ L of substrate freshly made before use.
9. Incubate at room temperature for exactly 5 minutes.
10. Stop the reaction by adding 50 μ L of 3 M sulphuric acid.
11. Leave the plate for 10 minutes for colour stabilisation.
12. Measure the absorbance at 492 nm.
13. The concentrations of test samples were derived from a log/log standard curve.

2.5 AMIDOLYTIC SUBSTRATE ASSAYS

These were used to measure kallikrein-like and thrombin-like activities in supernatant plasma, as well as the ability of MV isolated from platelet concentrates to support the formation of prothrombinase complex.

General Principle

Amidolytic substrate assays are based on the use of synthetic peptide substrates which are covalently linked to a chromophore, p-nitroaniline (pNA). Proteases cleave the substrate with the liberation of pNA. The pNA released is measured using spectrophotometrically at 405 nm and the change in absorbance is proportional to the enzyme activity (kinetic determination). Alternatively the substrate is incubated for a set time and the absorbance is then measured (end-point determination).

2.5.1 Kallikrein-like activity

i. Principle

The activation of the contact system of coagulation system generates kallikrein, which can be measured by a synthetic substrate for kallikrein (S2302). However, this substrate is not only sensitive to plasma kallikrein but also to FXa, activated protein C, complement C1s and thrombin. Therefore the measured enzyme activity is called kallikrein-like activity (KLA).

ii. Reagents

1. 50 mmol/L Tris buffer pH 8.0 (Sigma-Aldrich Company Ltd, Dorset, UK)
2. Chromogenic substrate S2302 (Chromogenix AB, Mölndal, Sweden) reconstituted with distilled water to obtain a concentration of 2 mM.
3. Flat bottom polystyrene microtitre plate (Alpha Laboratories Ltd, Hampshire, UK)

iii. Methods

1. Dilute plasma 1 in 20 with Tris buffer.
2. Add 200 μ L diluted plasma into each well.
3. Add 50 μ L chromogenic substrate and read the absorbance immediately at 405nm to get a blank reading.
4. Incubate at room temperature for 2 hours and read the absorbance again.
5. KLA is calculated from the difference between the absorbance at 2 hours and blank.

2.5.2 Thrombin-like activity

i. Principle

The activation of coagulation system leads to the generation of thrombin which can be measured by a synthetic substrate for thrombin (S2238). This substrate is also sensitive to FXa, FXIa, APC, and kallikrein. Therefore the measured enzyme activity is

called thrombin-like activity (TLA).

ii. Reagents

1. 50 mmol/L Tris buffer pH 8.0 (Sigma-Aldrich Company Ltd, Dorset, UK).
2. Chromogenic substrate S2238 (Chromogenix AB, Mölndal, Sweden) reconstituted with distilled water to obtain a concentration of 2 mM.
3. A flat bottom polystyrene microtitre plate (Alpha Laboratories Ltd, Hampshire, UK).

iii. Methods

1. Dilute plasma 1 in 20 with Tris buffer.
2. Add 200 μ L diluted plasma into each well.
3. Add 50 μ L chromogenic substrate and read the absorbance immediately at 405nm to get a blank reading.
4. Incubate at room temperature for 2 hours and read the absorbance again.
5. TLA is calculated from the difference between the absorbance at 2 hours and blank.

2.6 ASSAY PERFORMANCE

To assess the performance of all assays used in this study, the coefficient of variation (CV) of intra- (within) and inter- (between) assays were calculated. The intra-assay CV was calculated from 10 measurements of a single sample on one occasion. The inter-assay CV was calculated from the measurement of a single sample on 10 different occasions over a period of at least 10 days. The intra- and inter-assay CVs for tests described in this chapter are shown in Table 2.1.

Table 2.1 Intra- and inter-assay coefficient of variations.

Tests	Level assayed	Intra-assay CV (%)	Inter-assay CV (%)
P-selectin on platelet membrane	13.5 %	8.9	NA
Plt-MV	$1970 \times 10^3/\text{mL}$	8.3%	NA
RBC-MV	$230 \times 10^3/\text{mL}$	7.6%	NA
WBC-MV	$36 \times 10^3/\text{mL}$	10%	NA
Soluble P-selectin	98 ng/mL	4.8	6.2
FXIIa	1.20 ng/mL	4.6	10.0
Prothrombin F1+2	0.6 nmol/L	5.6	12.5
TAT	2.3 $\mu\text{g/L}$	4.5	9.2
C3a	1753 ng/mL	7.3	12.8
Soluble annexin V	9.2 ng/mL	6.8	8.4
KLA*	$\text{OD}_{405\text{nm}} 114 \times 10^{-3}$	7.2	9.8
TLA*	$\text{OD}_{405\text{nm}} 178 \times 10^{-3}$	7.6	11.3
Prothrombinase activity	0.07 U/mL	3.1	8.7

NA-inter-assay CV cannot be measured as these parameters change over storage of samples.

*Results were expressed as the difference between the $\text{OD}_{405\text{nm}}$ at zero time and 2 hours.

2.7 COMPUTER HARDWARE AND SOFTWARE

A Compaq Presario 1060 was used to prepared this thesis. Data was initially organised using Excel 95 (Microsoft Corp.). Data analysis was performed using Excel 97 and GraphPad Prism 2.0. Routine manual checks were carried out to ensure that

spreadsheet and statistical package software transformation of data was correct. The exact nature of the statistical analysis used will be stated in each chapter.

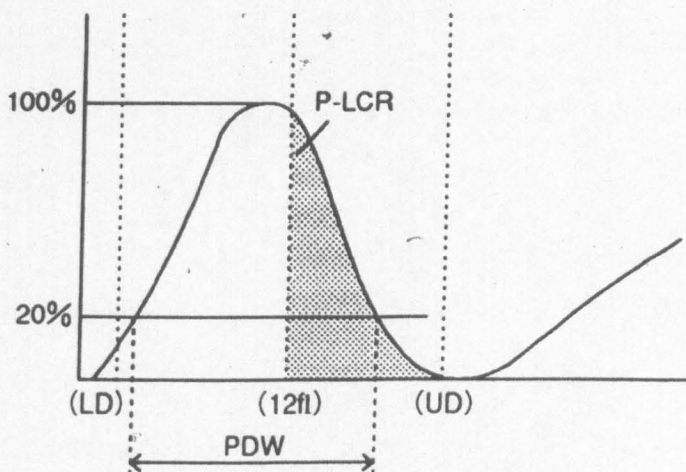
CHAPTER 3

PLATELET INDICES OF PLATELET CONCENTRATES

3.1 INTRODUCTION

Modern automated haematology analysers provide not only the platelet count but also platelet indices i.e. mean platelet volume (MPV), platelet distribution width (PDW), and platelet-large cell ratio (P-LCR). These indices are obtained from the principle shown in Figure 3.1 and described below.

Figure 3.1 PDW, P-LCR and MPV from an automated haematology analyser based on the direct current principle.



Particles with the size between the lower discriminator (LD) 2-6 fL and the upper discriminator (UD) 12-30 fL are counted as platelets. A fixed discriminator (FD) is set at 12 fL. PDW is the distribution width at the 20% level when the peak is equivalent to 100%. P-LCR is

the ratio of large platelets which fall in the range between the FD and the UD divided by the number of platelets falls in the range between LD and UD and multiplied by 100. MPV is calculated from the following formula:

$$\text{MPV (fL)} = \frac{\text{PCT}(\%)}{\text{PLT} (\times 10^3/\mu\text{L})} \times 1000$$

where PCT (plateletcrit or platelet volume ratio) is the weighted frequency of platelet at each MPV channel.

MPV can be used as a quantitative indicator of the presence of younger, larger platelets, which are more haemostatically active. MPV has been shown to be a useful marker in several pathological conditions (van der Loo 1997). For instance, MPV is increased in patients after myocardial infarction and can be used as a predictor of a further ischemic event. High MPV is also seen in patients with diabetes mellitus (Tschoepe 1991). Other platelet indices, P-LCR and PDW have also been shown to be useful diagnostic indicators. It has been demonstrated that MPV, P-LCR and PDW increase at the onset of disseminated intravascular coagulation (DIC). Furthermore, PDW can be used to differentiate between reactive thrombocytosis and thrombocytosis seen in myeloproliferative diseases, the latter giving a higher PDW (Osselaer 1997).

Reports on the application of platelet indices to blood components, in particular platelet concentrates, are limited. Early reports have suggested that different subpopulations of platelets measured by MPV may be recovered by different preparation methods (Brozovic 1992, Van der Planken 1999), and that MPV of stored platelets is lower than fresh (Vickers 1991, Racz 1992).

The difference in MPV before and after the addition of EDTA to a citrate sample, so called delta (d) MPV, has been shown to be correlated with various markers of the platelet storage lesion and platelet function e.g. pH, β -thromboglobulin, lactate

dehydrogenase, glyocalicin, hypotonic shock response (HSR), and platelet aggregation in response to ADP or ADP and collagen (Vickers 1991). PDW and P-LCR have also been shown to correlate with pH and HSR (Farrugia 1995).

The mechanism of EDTA induced platelet shape change leading to the increase in platelet size indices is unclear. Nevertheless, it has been hypothesised that the degree of shape change upon exposure to EDTA reflects the functional reserve of platelets (Seghatchian 1994). Platelets, which have already undergone activation, respond to EDTA to a lesser degree than those which are in resting state.

The objectives of this study were:

- i. To investigate whether leucocyte depleted platelet concentrates prepared by three different methods contain the same profiles of platelet size indices.
- ii. To investigate whether leucocyte depleted platelet concentrates prepared by three different methods contain the same functional reserve as measured by the response to EDTA.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of platelet concentrates

i. Leucocyte filtered pooled buffy coat derived platelets

Whole blood (450 ± 45 mL) was collected into top and bottom packs containing 63 mL of citrate-phosphate-dextrose anticoagulant (PL146, Baxter Healthcare, Deerfield, Illinois, USA). Buffy coats were prepared using a 'hard spin' (Beckman centrifuge J6MC, 3400 rpm, 11.5 min, 22°C) and kept overnight at 22°C. For each unit of platelet

concentrate, 4 units of ABO identical buffy coats and 1 unit of plasma from 1 of the 4 buffy coat donors were pooled together on the day following collection. An in-process leucocyte removal filter (Autostop BC, Pall Biomedical, Portsmouth, UK) was connected to the pool. Platelets were harvested using a 'soft spin' (1850 rpm, 5 min, 22°C).

ii. Apheresis platelets

Platelets collected by Haemonetics MCS+ LD were leucocyte depleted by passing through a leucocyte removal filter (LRF6H, Pall Biomedical, Portsmouth, UK) during the last return cycle (detail described in Chapter 1). The detailed principle of Cobe LRS apheresis system was previously described in Chapter 1.

Ten units of platelet concentrates prepared by each method were tested on day 1, the earliest time a product can be issued for clinical use. This also allowed direct comparison with buffy coat derived platelets. Each unit was tested again at the end of the 5-day storage shelf life. To maintain the sterility of the products, samples were taken in a close system by connecting to a Transfer Pack® (Baxter Healthcare, Deerfield, IL, USA), using a Sterile Connecting Device® (Terumo, Leuven, Belgium).

3.2.2 pH and leucocyte count

pH was measured on an AVL 9110 pH analyser (AVL Medical Instruments, Graz, Austria). Leucocyte counts were measured by flow cytometry as described in Chapter 2.

3.2.3 Platelet count and platelet indices

An automated haematology analyser, Sysmex SE-9000 (TOA Medical Electronics Co. Ltd, Kobe, Japan), was used. The detail of this instrument was described in Chapter 2.

For each platelet unit, 2 mL of sample were placed in a 4 mL plain polystyrene tube tightly capped, and 250 µL in a 1.8 mL dipotassium ethylenediamine tetraacetic

acid (EDTA) tube (L.I.P. Equipment & Services Ltd., West Yorkshire, UK). The final concentration of EDTA was 25.4 mmol/L. The latter was gently mixed by rolling the tube manually for 5 seconds. Both paired samples were kept static at room temperature for 1 hour and then analysed on the haematology analyser at the same time. The difference of the results between the paired samples was calculated using the formula below: (Seghatchian 1994)

$$\text{dMPV} = \text{MPV}_{\text{EDTA}} - \text{MPV}_{\text{citrate}}$$

dPLT, dP-LCR and dPDW were calculated in the same fashion.

3.2.4 Statistical analysis

Paired t-test and ANOVA test were used to compare two and more than two sets of results, respectively. Pearson test was used to analyse the correlation between results. Results of the three types of platelets were compared between groups and within each group at day 1 and day 5.

3.3 RESULTS

3.3.1 pH and leucocyte count

There was no significant difference in pH between groups on day1, but on day 5 both MCS ($p<0.01$) and Cobe ($p<0.001$) were lower than Autostop filtered BC-PC (Table 3.1). All units were within the acceptable range (6.4-7.4) (Guidelines for the Blood Transfusion Services in the United Kingdom 1998). Residual leucocyte counts were less than 5×10^6 /unit in all products.

Table 4.1 pH of all three groups.

Mean \pm SD

	Autostop	MCS	Cobe
Day 1	7.10 \pm 0.03	7.14 \pm 0.08	7.11 \pm 0.12
Day 5	7.27 \pm 0.03	7.14 \pm 0.11	7.16 \pm 0.06

3.3.2 Platelet count

In all cases the platelet counts were higher in EDTA samples than citrate ($p<0.001$), as platelet aggregates which tend to form in citrate samples are dispersed by EDTA. Therefore, the results of platelet count in EDTA samples were used for statistical analysis on the basis that they represented more accurately the number of platelets in each product. MCS platelets showed high variability between units. The platelet counts of Cobe products were significantly higher than Autostop ($p<0.001$) and MCS ($p<0.01$) (Figure 3.2). However, the platelet yields (platelet count \times volume of each unit) were not statistically different. No difference was seen in the platelet counts within the same type of products tested on day 1 and day 5.

The difference between platelet counts of paired citrate and EDTA samples (dPLT), reflecting the amount of reversible aggregates, were significantly higher in Cobe than Autostop ($p<0.001$) (Figure 3.3). No difference in dPLT was found within the same product over storage. A significant correlation between platelet count and dPLT was observed in Autostop ($r=0.6113$, $p=0.0302$) and MCS ($r=0.7987$, $p=0.0028$) but not in Cobe (Figure 3.4).

Figure 3.2 Platelet count (PLT) in citrate (C) and EDTA (E) samples of Autostop filtered pooled buffy coat derived, MCS+ and Cobe apheresis platelets on day 1.

Mean ■ and SD. E were higher than C in all cases. Cobe were higher than Autostop ($p<0.01$) and MCS ($p<0.001$).

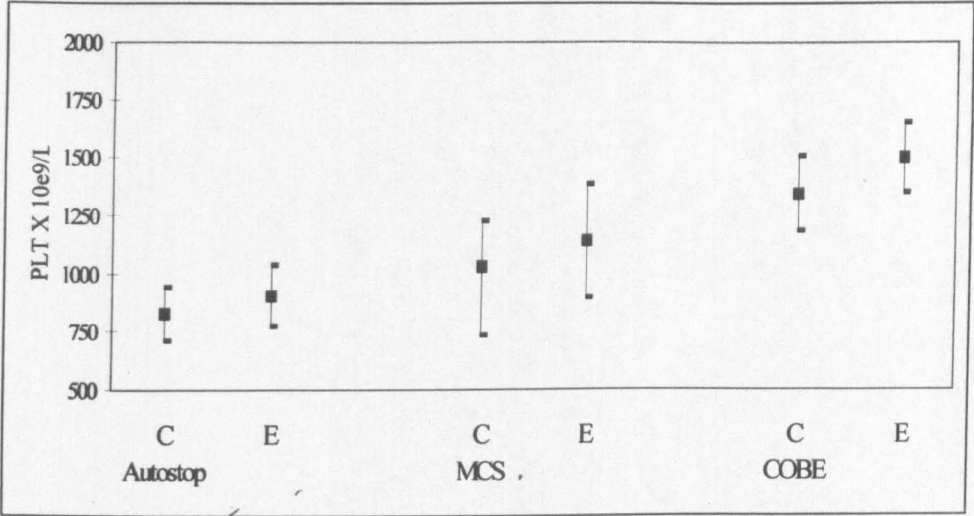


Figure 3.3 dPLT in all four groups on day 1.

Cobe were higher than Autostop filtered BC-PC ($p<0.001$).

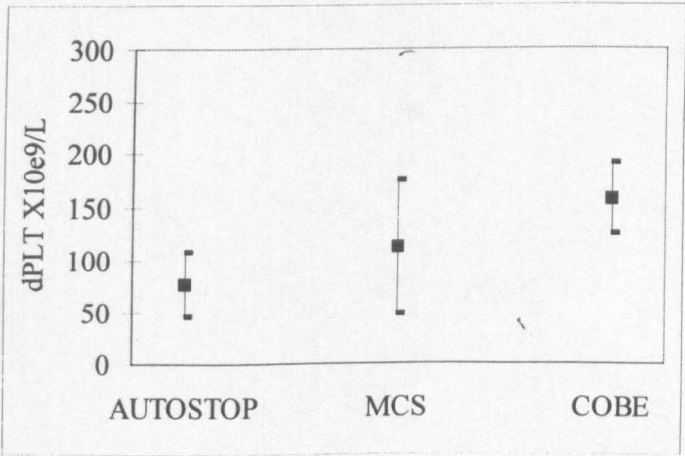
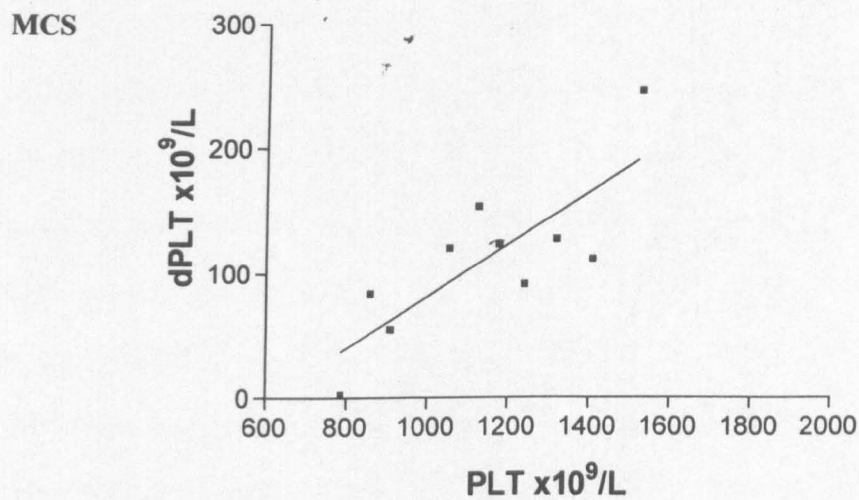
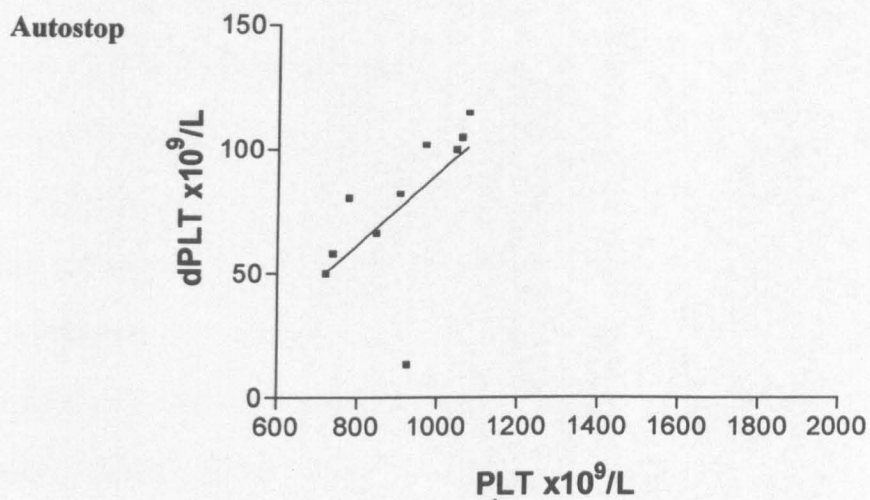


Figure 3.4 Correlation between dPLT and platelet count (PLT) in Autostop and MCS on day 1.

Top-Autostop, bottom-MCS. Autostop $r=0.6113$, $p=0.0323$, MCS $r=0.7987$, $p=0.0028$.



3.3.3 MPV, P-LCR and PDW

There was no significant difference in the MPV of citrate samples between the three groups either immediately after production or on storage. However, they responded to EDTA to various degrees, resulting in differences in dMPV. Figure 3.5 shows the change in platelet size distribution upon the addition of EDTA. On day 1, both Autostop ($p<0.01$) and MCS ($p<0.05$) platelets gave higher dMPV than Cobe. On day 5, all three groups were different from each other ($p<0.01$, 0.001, and 0.01 for Autostop vs MCS, Autostop vs Cobe, and MCS vs Cobe, respectively). (Figure 3.6)

The MPV within the same group did not change on storage. The dMPV significantly increased on storage in all groups ($p<0.0001$, $=0.0351$, and $=0.0005$ for Autostop, MCS, and Cobe, day 1 vs day 5, respectively) (Figure 3.7). No correlation was found between MPV and dMPV.

Similar patterns to MPV and dMPV were seen with P-LCR, dP-LCR, PDW, and dPDW. No difference was found in P-LCR and PDW between day 1 and day 5 either within the same type of platelets or between the three types. On day 1, Cobe dP-LCR was lower than Autostop ($p<0.001$). On day 5 both MCS and Cobe were lower than Autostop ($p<0.05$ and 0.001, respectively) (Figure 3.8). All three types showed higher dP-LCR on day 5 than day 1 ($p<0.001$, $=0.0147$, $=0.0008$, for Autostop, MCS, and Cobe day 1 vs day 5, respectively) (Figure 3.9).

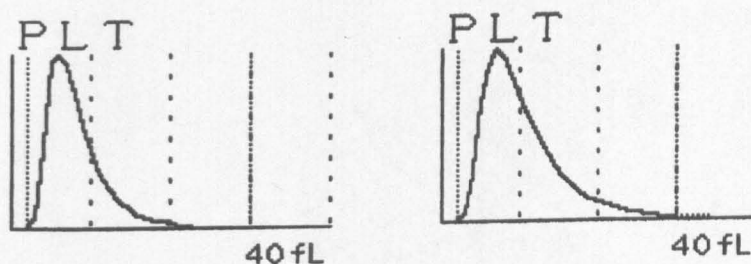
On day 1 dPDW of Cobe was lower than Autostop ($p<0.01$). On day 5, both Cobe and MCS were lower than Autostop ($p<0.01$ and 0.001, respectively). (Figure 3.10) All three types of platelets showed higher dPDW on day 5 than day 1 ($p=0.0002$, 0.0225, and 0.0009 for Autostop, MCS, and Cobe day 1 vs day 5, respectively). (Figure 3.11)

Significant correlations between platelet count and MPV ($r=0.7056$, $p=0.0226$) or PDW ($r=0.6991$, $p=0.0245$), were found only in Autostop, but not in either type of

apheresis platelets (Figure 3.13). The correlation between platelet count and P-LCR was not significant ($r=0.5583$, $p=0.0935$). MPV and PDW showed high degree of correlation ($r=0.9944$, $p<0.0001$) as shown in Figure 3.14, whereas no correlation was found between either of these indices with P-LCR.

Figure 3.5 Change in platelet size distribution after adding EDTA.

Left-citrate sample, right-EDTA sample. PLT-platelet count, MPV-mean platelet volume, P-LCR-platelet large cell ratio, PDW-platelet distribution width.



PLT $956 \times 10^9/L$

MPV 8.2 fL

P-LCR 13.1 %

PDW 9.5 fL

PLT $1043 \times 10^9/L$

MPV 9.5 fL

P-LCR 23.5 %

PDW 12.8 fL

Figure 3.6 MPV and dMPV of three types of platelets.

Left panels-MPV, right-dMPV, top-day 1, bottom-day 5. No differences in MPV was seen between products. dMPV day 1, Autostop and MCS were higher than Cobe ($p<0.01$, 0.05 , respectively), on day 5 all were significantly different from each ($p<0.01$, 0.001 , and 0.01 for Autostop vs MCS, Autostop vs Cobe, and MCS vs Cobe, respectively).

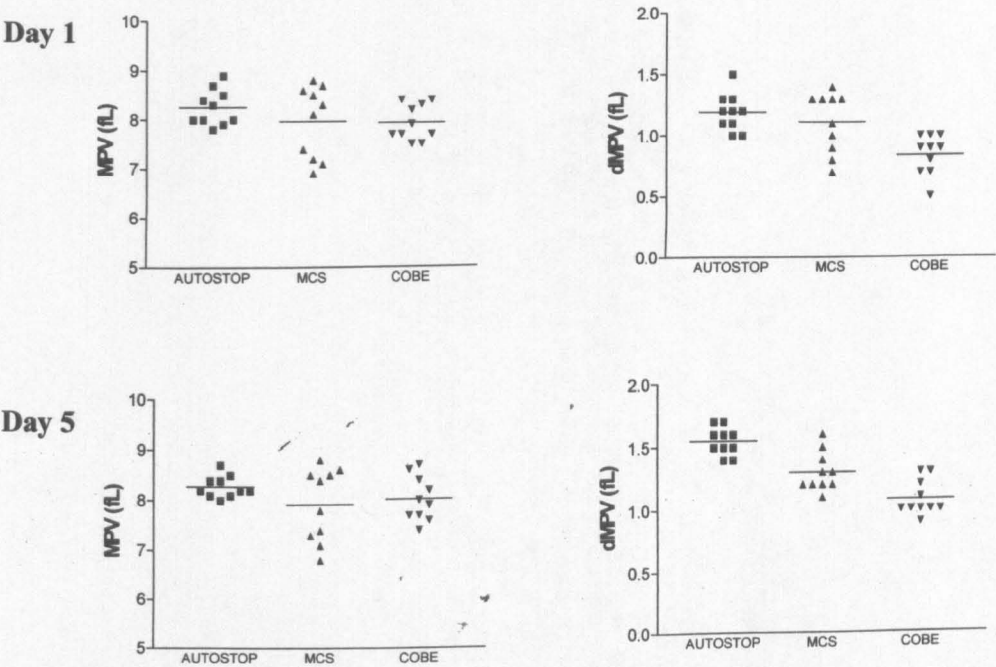


Figure 3.7 Changes in MPV and dMPV of each type of platelets on storage.

Top-Autostop, middle-MCS, bottom-Cobe, left-MPV, right-dMPV. No significant difference was seen in MPV between day 1 and day 5, whereas dMPV was significantly higher on day 5 than day 1 ($p<0.0001$, $=0.0351$, and $=0.0005$ for Autostop, MCS, and Cobe, day 1 vs day 5, respectively).

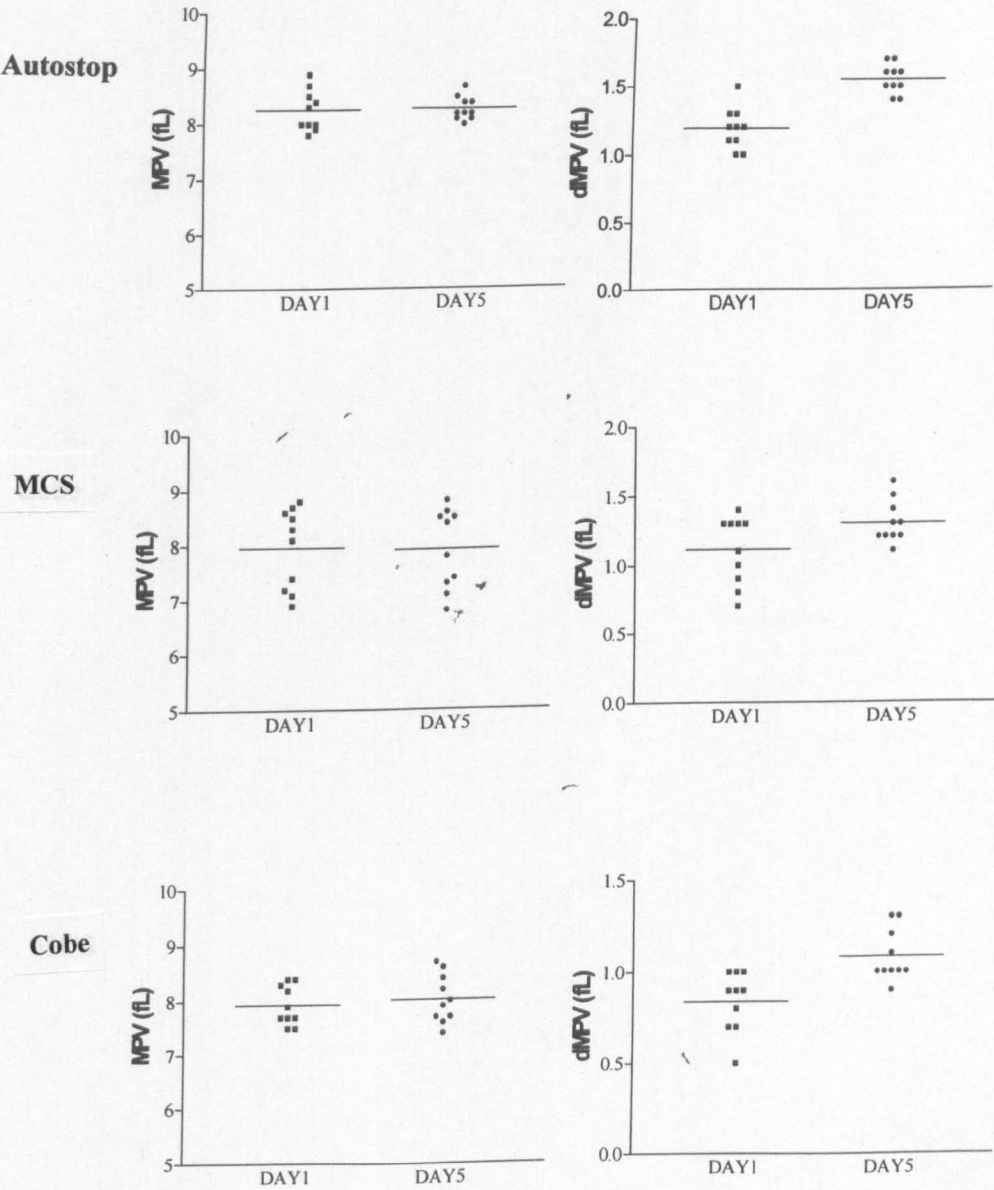


Figure 3.8 P-LCR and dP-LCR of three types of platelets.

Left panels P-LCR, right dP-LCR, top-day 1, bottom-day 5. No difference was found in P-LCR on day 1 or day 5. On day 1, Cobe dP-LCR was lower than Autostop ($p<0.001$). On day 5 both MCS and Cobe were lower than Autostop ($p<0.05$ and 0.001 , respectively).

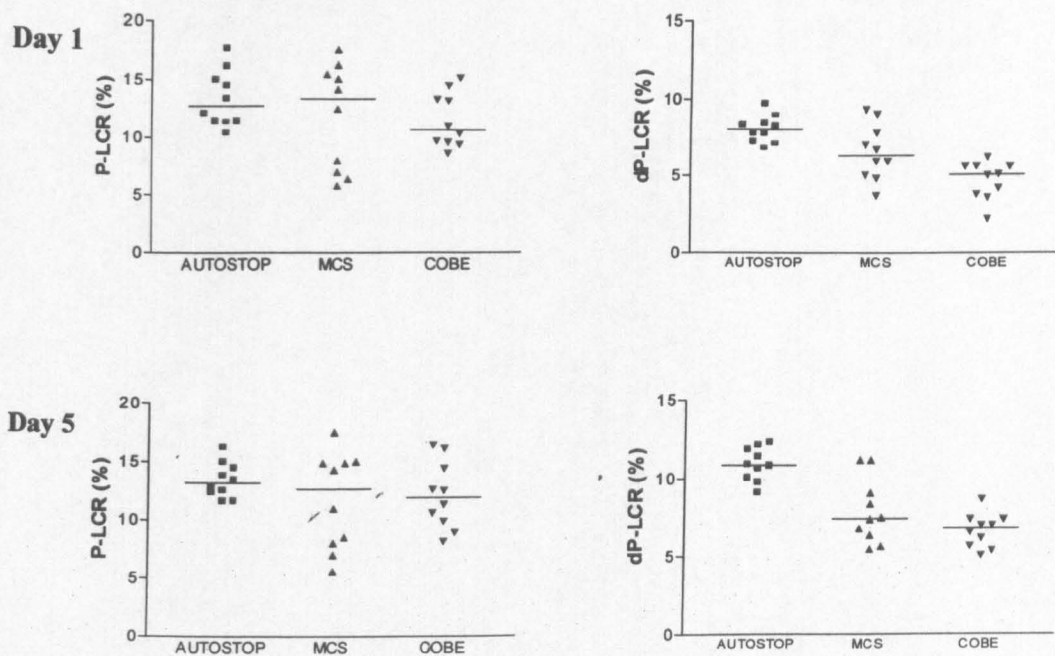


Figure 3.9 Changes in P-LCR and dP-LCR of each type of platelets on storage.

Left panels P-LCR, right dP-LCR, top-Autostop, middle-MCS, bottom-Cobe. No difference was found in P-LCR between day 1 and day 5. All three types showed higher dP-LCR on day 5 than day 1 ($p < 0.001$, $=0.0147$, $=0.0008$, for Autostop, MCS, and Cobe day 1 vs day 5, respectively).

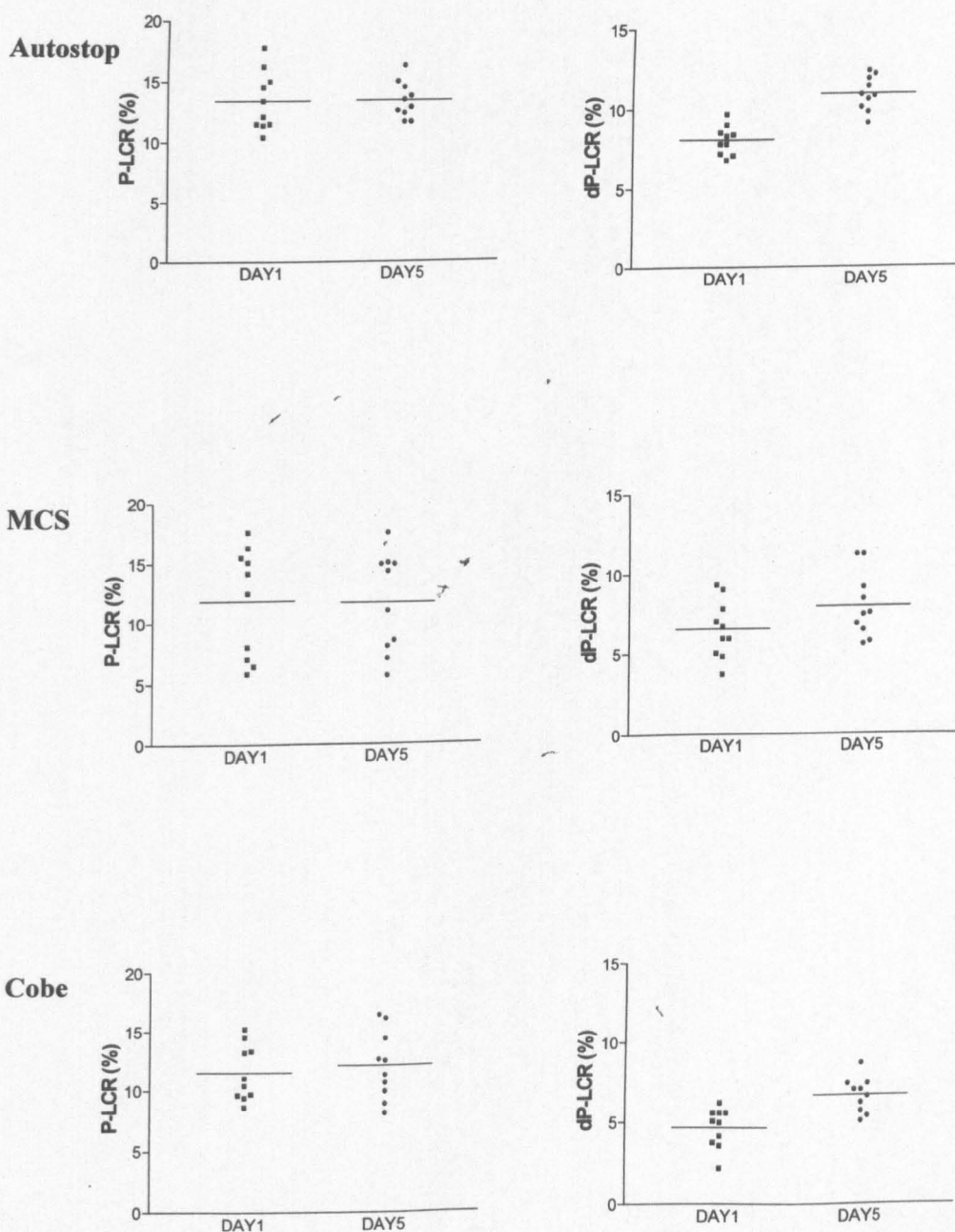


Figure 3.10 PDW and dPDW of three types of platelets.

Left panels PDW, right dPDW, top-day 1, bottom-day 5. No difference was found in PDW on day 1 or day 5. On day 1, Cobe dPDW was lower than Autostop ($p<0.01$). On day 5 both MCS and Cobe were lower than Autostop ($p<0.05$ and 0.001 , respectively).

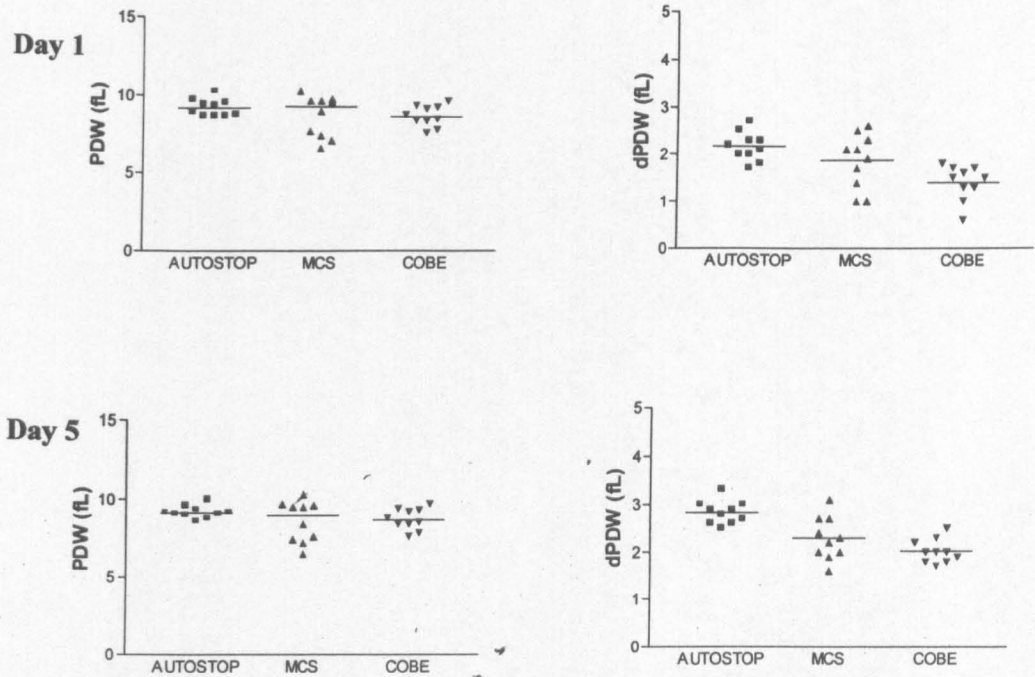


Figure 3.11 Changes in PDW and dPDW of each type of platelets on storage.

Left panels-PDW, right-dPDW, top-Autostop, middle-MCS, bottom-Cobe. No difference was found in PDW between day 1 and day 5. All three types showed higher dPDW on day 5 than day 1 ($p=0.0002$, 0.0225 , and 0.0009 for Autostop, MCS, and Cobe day 1 vs day 5, respectively).

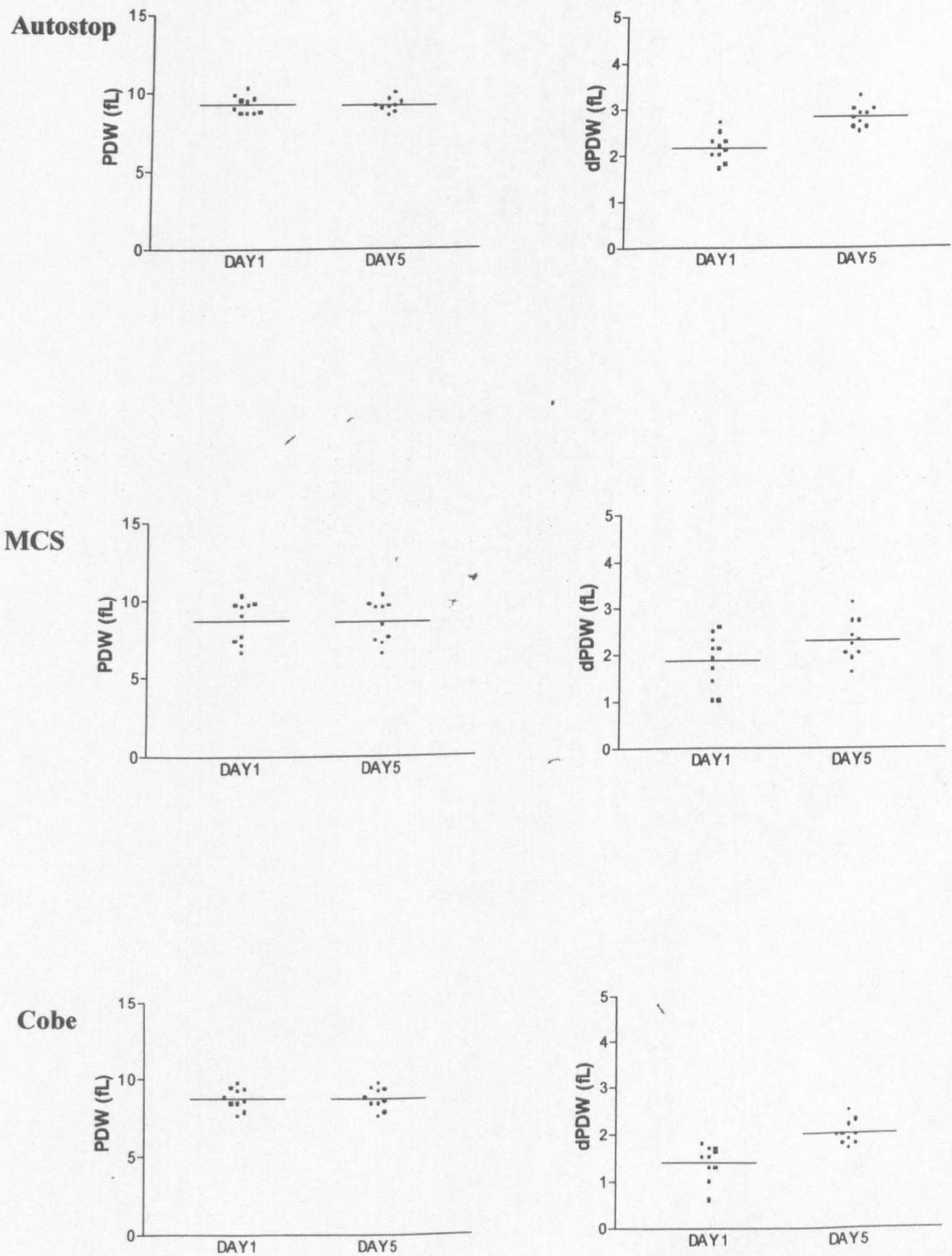


Figure 3.12 Correlation between platelet count and MPV or PDW in Autostop on day 1.

Top-PLT VS mpv, bottom-PLT vs PDW. Significant correlations were seen in only Autostop ($r=0.7056$, $p=0.0226$, and $r=0.6991$, $p=0.0245$, for PLT vs MPV and PDW, respectively).

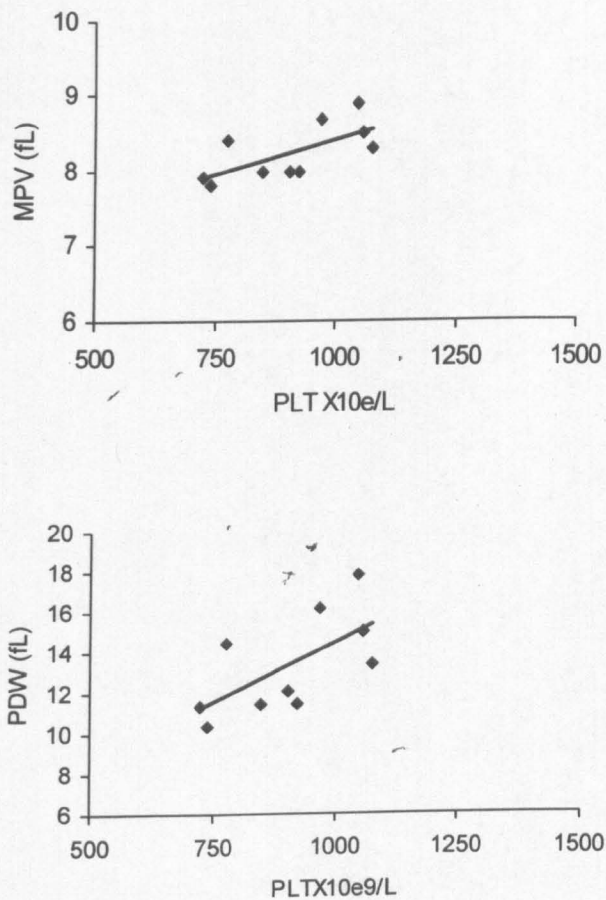
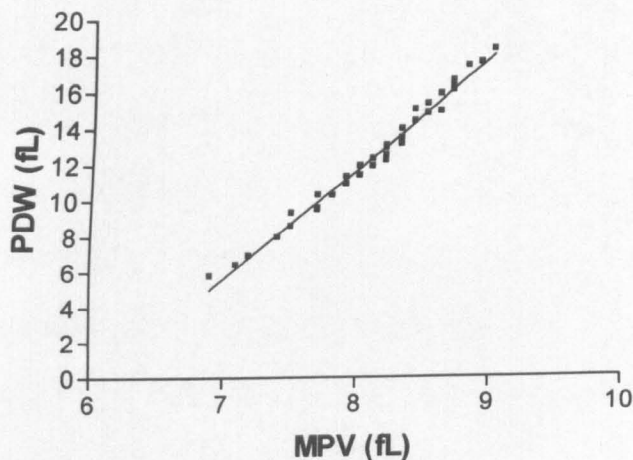


Figure 3.13 Correlation between MPV and PDW

$r=0.9944$, $p<0.0001$, all day 1 data pooled.



3.4 DISCUSSION

All platelet concentrates prepared by the three methods, pooled buffy coat derived, Autostop filtered, MCS+LD and COBE LRS apheresis, met the UK specification for leucocyte depleted platelet concentrates, i.e. platelet yield 240×10^9 /unit, residual leucocyte content less than 5×10^6 /unit, and pH at the end of 5-day storage between 6.4 to 7.4 (Guidelines for the Blood Transfusion Services in the United Kingdom 1998). Minimal changes in pH i.e. slightly increase were observed on storage as the third generation storage bags currently in use allowed good gas exchange.

Citrate anticoagulants are routinely used in the preparation of platelet concentrates for transfusion. Using this anticoagulant, the concentration of Ca^{2+} is reduced from 1-2 mmol/L to 46-74 $\mu\text{mol/L}$ (Bode 1989). It has been suggested that the level of Ca^{2+} present in citrated anticoagulated platelet concentrates is sufficient to activate some Ca^{2+}

dependent proteases in platelets and plasma resulting in platelet activation, and thrombin generation (Bode 1989). These may lead to the formation of loose platelet aggregates which can be dispersed by the addition of EDTA, a strong chelating agent, leading to approximately 10 % higher platelet count on average. A previous report has shown that platelet counts in EDTA added samples are 6-22% higher than citrate (McShine 1990). The higher dPLT in Cobe product was probably secondary to the fact that this product contained higher platelet count, as the ratio of dPLT to total platelet count were approximately 1:10, the same as the other two products. The clinical significance of these dispersible platelet aggregates presence in platelet concentrates is unknown. It is possible that they may adhere to the bags when kept without agitation during transport or transfusion, leading to some loss of platelets.

Platelet size indices (MPV, PDW, and P-LCR) did not show any difference either between the three types of platelets or within the same type on storage. This was because no gross difference in pH occurred as these parameters had been shown to be correlated with pH. A recent report using the third generation storage bag has also demonstrated that the MPV remains unchanged during storage, but no data on pH is presented (Van der Planken 1999).

The correlation between platelet count and MPV or PDW seen in only Autostop filtered, buffy coat derived platelets may be explained by the preparation method. During the first spin in the buffy coat method, the platelets with relatively larger size are sedimented near the red blood cell/leucocyte layer. For the production of platelet concentrates, to recover maximum platelets this population had to be extracted into the final product, whereas in both apheresis preparations this was achieved by increasing the volume processed.

Upon the addition of EDTA, the three types of products behaved differently.

Cobe showed the least changes in platelet size indices, whereas Autostop and MCS were equivalent. The difference between Cobe and Autostop was greater than between Cobe and MCS, or MCS and Autostop. This might be related to the basic principles employed by the three methods. Both MCS and Autostop shared a common principle, in which a buffy coat was prepared as an intermediate product and the final product was passed through a leucocyte removal filter. By contrast, platelets prepared by Cobe LRS apheresis method required a platelet rich plasma as an intermediate product before being leucodepleted in a conical chamber by a fluidised particle bed principle.

In the early days of development of platelet concentrates for transfusion, when EDTA was used as an anticoagulant, it was found that the viability of platelets was markedly reduced as compared to those collected into acid citrate dextrose (ACD) (Aster 1965). Previous study using electron microscopy showed that EDTA induced platelet swelling (Brozovic 1991). Furthermore, α -granule release with P-selectin expression was demonstrated in platelets prepared from EDTA anticoagulant (Golanski 1996, Holme 1997). The mechanism of EDTA induced changes in platelets is unclear. Nevertheless, it has been suggested that EDTA induces conformation changes of platelet membrane structural protein as well as triggering the tyrosine kinase signal transduction pathway via non-specific receptors (Golanski 1996).

Although leucocyte depleted platelet concentrates prepared by all three methods have the same platelet size indices profile when using citrate samples, they responded to the addition of EDTA in different fashions. This indicated that platelets recovered by the three preparation methods contained different functional reserve. It remains to be elucidated whether they have the same haemostatic effectiveness in vivo. Nevertheless, it could be hypothesised that platelets with a higher level of functional reserve were

desirable, as they would respond more effectively to stimuli when required.

CHAPTER 4

MICROVESICLES IN POOLED BUFFY COAT-DERIVED PLATELET CONCENTRATES

4.1 INTRODUCTION

During the preparation of platelet concentrates (PCs) from pooled buffy coats, platelets are kept in contact with a high number of leucocytes overnight at 20-22°C. The proteolytic enzymes released by leucocytes such as elastase may cause proteolysis of the platelet membrane (Brower 1985, Sloan 1990). This may lead to platelet microvesiculation. It is not known whether the process of leucocyte filtration removes the pre-formed microvesicles (MV) and/or whether the leucocyte filtration process might lead to shear-induced platelet or red cell microvesiculation. In addition, different types of filters made from different materials, namely positively charged polyester, negatively charged polyester and neutrally charged polyurethane, may not have the same effects on platelet microvesiculation, although they appear to be equivalent in terms of leucocyte removal. The types of plastic, plasticisers and the volume/surface area of storage bags provided by various manufacturers are also different from each other. These may lead to differences in MV formation during storage.

It should be noted that for comparison between various filters, it is important to eliminate inter-donor variability by using a pooled study design. To date comparisons between filters have used either platelet concentrates from different donors or have pooled and re-split several units of pre-manufactured platelets. In this study, to reflect the procedure commonly employed in routine production of platelet concentrates in Europe,

pooled buffy coats from the same donors in combination with in-process filters were used. This approach allows comparison of three commercially available in-process filter/storage bag combinations and unfiltered controls while minimising inter-donor variability.

The aims of this study were:

- i. To investigate whether leucocyte filtration activates platelets.
- ii. To investigate whether leucocyte filtration removes or generates platelet-derived and red cell-derived microvesicles (Plt-MV, RBC-MV) and whether various filters made from different materials have the same effects.
- iii. To investigate whether leucocyte depleted platelets prepared by various filters undergo the same degree of microvesiculation upon storage.

4.2 MATERIAL AND METHODS

4.2.1 Filters and platelet storage bags

Three in-process leucocyte removal filter/PC storage bag combinations currently provided by manufacturers were used:

- i) Autostop BC/CLX (Pall Biomedical, Portsmouth, UK)
- ii) Sepacell PLX5/PL2410 (Asahi Medical Co./Baxter Healthcare, Lessine, Belgium)
- iii) Imugard III-PL 4P/Teruflex (Terumo Europe NV, Leuven, Belgium).

In addition, Cobe platelet sample bags (Cobe Laboratories Ltd., Denver, USA) were used to store a sample from each unit.

The compositions and structures of filters were described previously in Chapter 1, Table 1.7. The volume, plastic and plasticisers used in the storage bags are shown in Table 4.1 below.

Table 4.1. Synthetic material used in three filters, their structures, plastic, plasticiser and volume of various storage bags

Filter/Bag	Bag Plastic	Bag Plasticiser	Bag Volume (L)
Autostop BC/CLX	polyvinyl chloride	trioctyl trimellitate	1.5
Sepacell PLX5/PL2410	polyolefin	—	1.3
Imugard III/Teruflex	polyvinyl chloride	di n-decylphthalate	1.0
Control/CLX	polyvinyl chloride	trioctyl trimellitate	1.5
Cobe sample bag	polyvinyl chloride	butyryl tri-n-hexyl citrate	55 mL

4.2.2 Preparation of platelet concentrates

Whole blood (450 ± 45 mL) was collected into bottom and top packs containing 63 mL of citrate-phosphate-dextrose anticoagulant (PL146, Baxter Healthcare). Buffy coats were prepared using a 'hard spin' (Beckman centrifuge J6MC, 3400 rpm, 11.5 min, 22°C) and kept overnight at 22°C. For each set of experiments, 4 identical pools of buffy coats were prepared on the day following collection from 16 ABO identical buffy coats and 4 units of plasma from the same donors (Figure 4.1). Each pool was connected to one of the three in-process filters/storage bags described above. The control unfiltered pool was connected directly to a CLX storage bag (Pall Biomedical), the bag in routine use in the National Blood Service-London and South East Zone. Platelet concentrates were prepared using a 'soft spin' (1850 rpm, 5 min, 22°C). Using a sterile technique, a

sample from each unit was taken for testing after preparation by docking on a Transfer Pack® (Baxter Healthcare). Twenty-five mL of each unit were also taken into a Cobe sample bag (Cobe Laboratories Ltd., Denver, USA) for storage. Both Cobe sample bags and the original bags provided by the manufacturers were kept on a flat bed platelet agitator (Helmer, Baxter Healthcare, Illinois, USA) at 22°C. Samples were taken again on day 5. In total 10 sets of pools were analysed.

4.2.3 pH, platelet and leucocyte counts

pH was measured on an AVL 9110 pH analyser (AVL Medical Instruments, Graz, Austria). Platelet counts were measured on an automated cell counter (Sysmex SE9000, Kobe, Japan). Leucocyte counts were measured by flow cytometry as described in Chapter 2.

4.2.4 Platelet activation

The expression of P-selectin on platelet surface membrane was measured by flow cytometry, and P-selectin in the supernatant plasma (soluble P-selectin) was measured by ELISA as described in Chapter 2.

4.2.5 Platelet and red cell-derived microvesicles

These were measured by flow cytometry as described in Chapter 2.

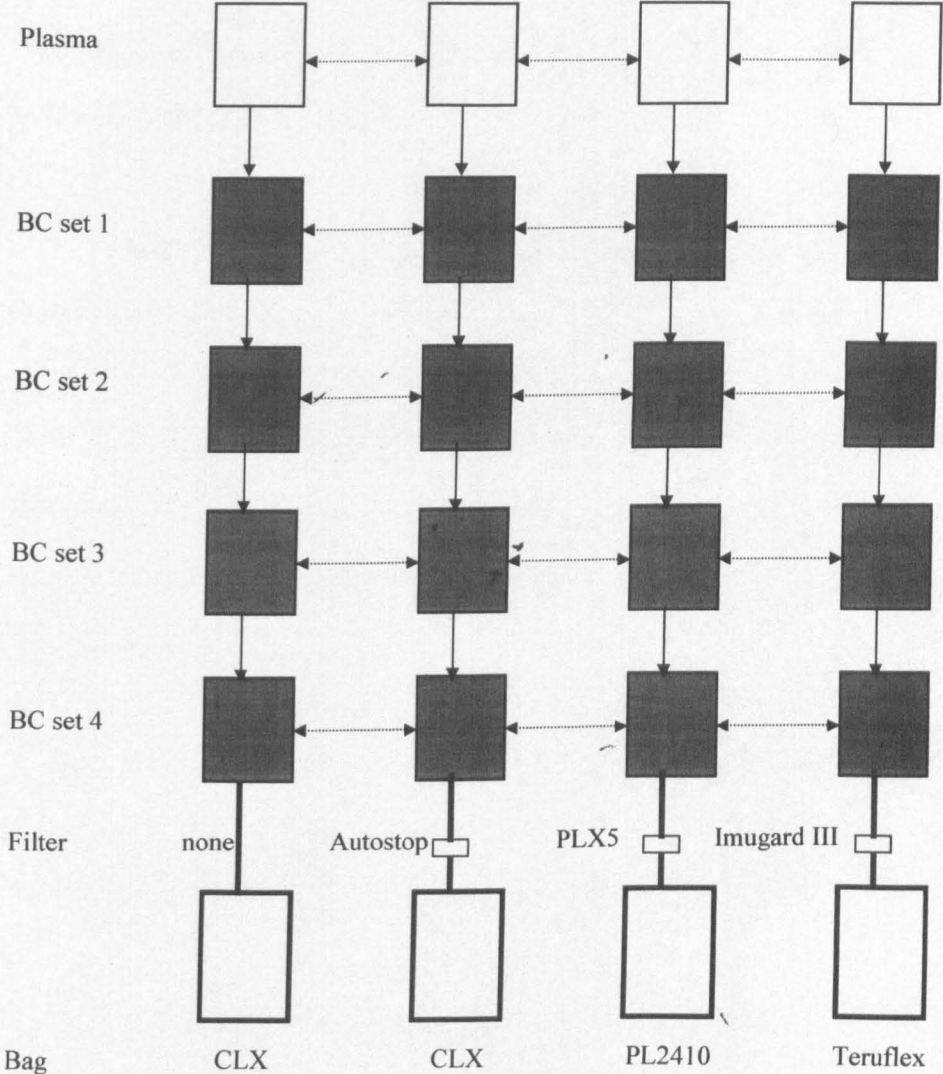
4.2.6 Statistical analysis

Friedman ANOVA test was used for a multiple group comparison i.e. the unfiltered and the three types of filtered platelet concentrates. Spearman test was used to analyse the correlation between results.

Figure 4.1 Preparation of pooled buffy coats

Each set of experiments consists of 16 units of buffy coats (BC) of the same ABO group and 4 plasma units from the same donors.

- ↔ step 1. Pool 4 units of plasma and equally subdivide into the 4 original bags. For each set of BC, pool 4 units together and equally subdivide into the 4 original bags.
- ↓ step 2. Pool 1 unit of plasma and 4 units of BC (one from each set).
- ↓ step 3. Connect each of the 4 pools of BC to one of the filter/storage bag combinations.



4.3 RESULTS

4.3.1 Volume, platelet and leucocyte contents and pH (Table 4.2)

No significant difference was found in the volume of pooled buffy coats and platelet concentrates between the four types of products. The platelet yields in control, Autostop and Imugard III filtered platelets were equivalent. PLX5 filtered platelets showed 10% lower platelet yields than three other groups (not statistically significant). All filtered units contained less than 1×10^6 leucocytes. pH was within acceptable range (6.4-7.4) both on day 1 and day 5.

Table 4.2 Volume of pooled buffy coats (BCs) and platelet concentrates (PCs), platelet (Plt) yield, leucocyte (WBC) content and pH, shown as median (range). n = 10

	Control	Autostop	PLX5	Imugard III
BCs (mL)	515 (434-558)	512 (469-577)	518 (469-566)	519 (501-563)
PCs (mL)	346 (291-364)	336 (308-371)	349 (302-381)	335 (312-368)
Plt ($\times 10^9$ /unit)	310 (289-343)	314 (239-388)	284 (228-372)	311 (249-365)
WBC ($\times 10^6$ /unit)	45 (30-74)	*	*	*
pH				
day 1	7.13 (7.07-7.16)	7.10 (7.05-7.15)	7.12 (7.11-7.18)	7.12 (7.07-7.18)
day 5	7.25 (7.24-7.28)	7.26 (7.23-7.31)	7.33 (7.28-7.37)	7.23 (7.20-7.27)

*Lower than the detection limit of 1 WBC/ μ L, equivalent to approximately less than 4×10^5 /unit.

4.3.2 P-selectin on platelet membrane and in plasma supernatant

On day 1, the levels of P-selectin on the platelet membrane and in the supernatant plasma in all four types of platelets were equivalent. Both were increased by 3-4 fold on day 5 in all four types of platelets (all $p < 0.05$), with Imugard III expressing significantly

lower P-selectin on platelet membrane than control platelets ($p<0.05$) (Figure 5.2). The differences between groups were greater with soluble P-selectin, with Imugard III giving significantly lower values than control ($p<0.001$) and Autostop ($p<0.001$) (Figure 5.3). A significant correlation was observed between P-selectin on platelet membrane and in the supernatant plasma ($r=0.8035$, $p<0.0001$). (data from all four groups day 1 and day 5 pooled, Figure 5.4).

No significant difference in soluble P-selectin was found between the four types of platelets in the Cobe sample bag.

No correlation was found between platelet count and platelet activation measured by both assays.

4.3.3 Microvesicles

There was considerable variability between pools in levels of Plt-MVs and RBC-MV. On day 1, Plt-MV numbers were not significantly different between groups, implying that leucocyte filtration did not generate or remove Plt-MV (Figure 4.5). By day 5 Imugard III showed significantly lower values than control ($p<0.05$) and Autostop PCs ($p<0.05$). Plt-MV increased during storage in all 4 groups, although no correlation was found between Plt-MV numbers on day 1 and day 5.

No statistically significant difference was seen in the levels of Plt-MV in different types of PCs stored in the Cobe sample bag.

The levels of RBC-MV in PLX5 and Imugard III were lower than control and Autostop (all $p<0.01$) on day 1, suggesting removal by the two former filters (Figure 4.6). Day 5 values in control units were increased from day 1, but in all three filtered PCs were unchanged. All filtered platelets showed lower values than control but only PLX5 and Imugard III reached statistical significance ($p<0.01$). PLX5 was also lower than Autostop ($p<0.05$)

Similar results of RBC-MV were observed in all platelets stored in the Cobe sample bag.

No correlation was found between platelet count and Plt-MV on both day 1 and day 5. Furthermore, no correlation was found between P-selectin on platelet membrane or soluble P-selectin and Plt-MV on day 1. However, on day 5 a low degree but significant correlation was observed between P-selectin on platelet membrane or soluble P-selectin and Plt-MV ($r=0.4215$, $p=0.0081$, and $r=0.5423$, $p=0.0002$, respectively). (Figure 4.7)

No correlation was found between Plt-MV and RBC-MV in filtered platelets, whereas unfiltered control showed significant correlation ($r=0.5714$, $p=0.0042$). (Figure 4.8)

Figure 4.2 P-selectin on platelet membrane on day 1 and day 5.

— Median. On day 5, Imugard III was significantly lower than control ($p<0.05$).

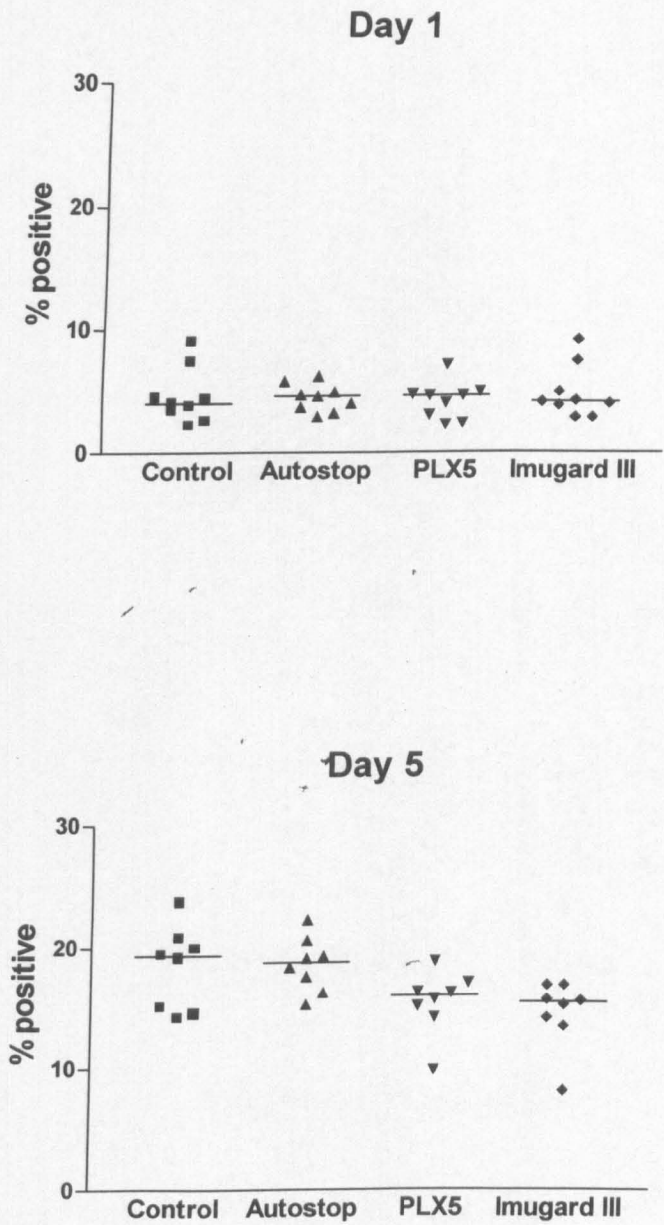


Figure 4.3 Soluble P-selectin on day 1 and day 5 in original and Cobe sample bags.

— Median. Day 5 in original bags Imugard III was significantly lower than control ($p<0.001$) and Autostop ($p<0.001$).

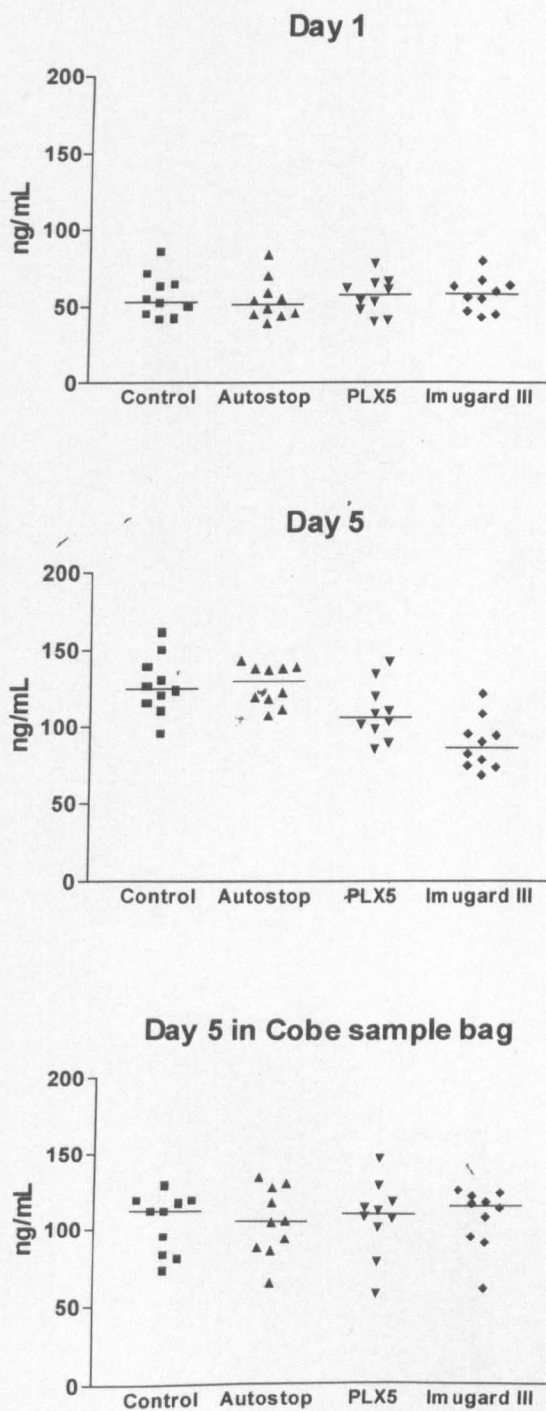


Figure 4.4 Correlation between P-selectin on platelet membrane and soluble P-selectin.

$r = 0.8035$, $p < 0.0001$, pooled data from all 4 groups day 1 and day 5.

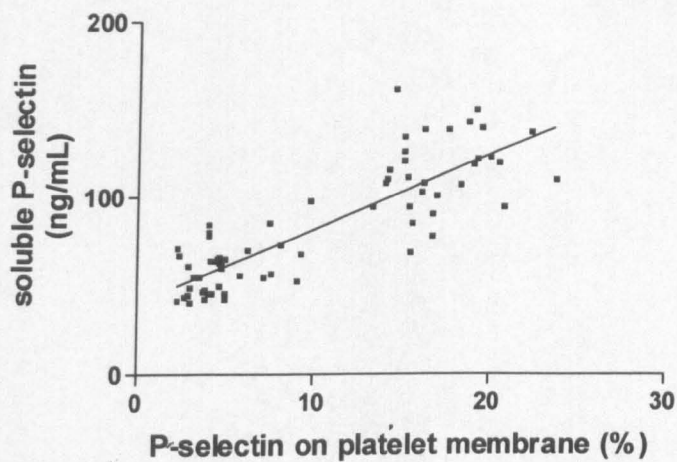


Figure 4.5 Plt-MV on day 1 and day 5 in original and Cobe sample bags.

— Median. By day 5 in original bags, Imugard III were significantly lower than control ($p<0.05$) and Autostop ($p<0.05$).

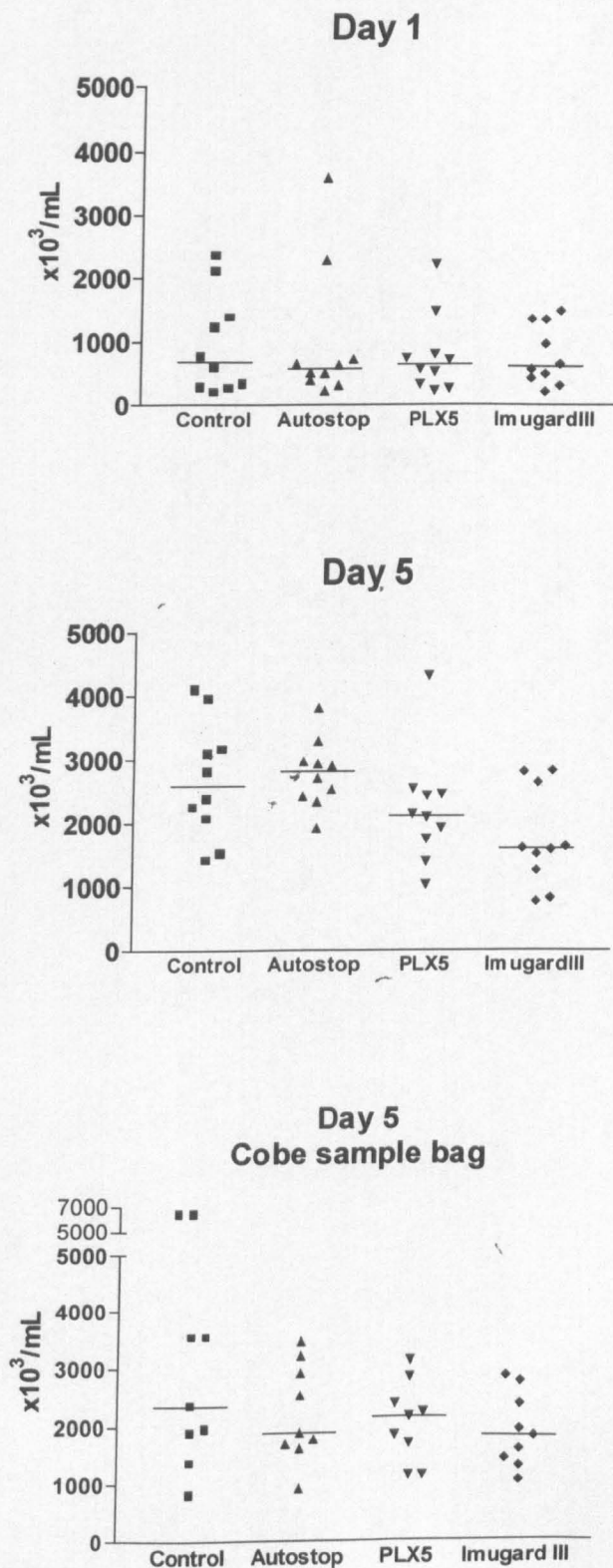


Figure 4.6 RBC-MV on day 1 and day 5 in original and Cobe sample bags.

— Median. Day 1, PLX5 and Imugard III were significantly lower than control ($p<0.01$) and Autostop ($p<0.01$). Day 5 in both original and Cobe sample bags, PLX5 and Imugard III were significantly lower than control values ($p<0.001$ PLX5 vs control, $p<0.01$ Imugard III vs control). PLX5 was also lower than Autostop ($p<0.05$).

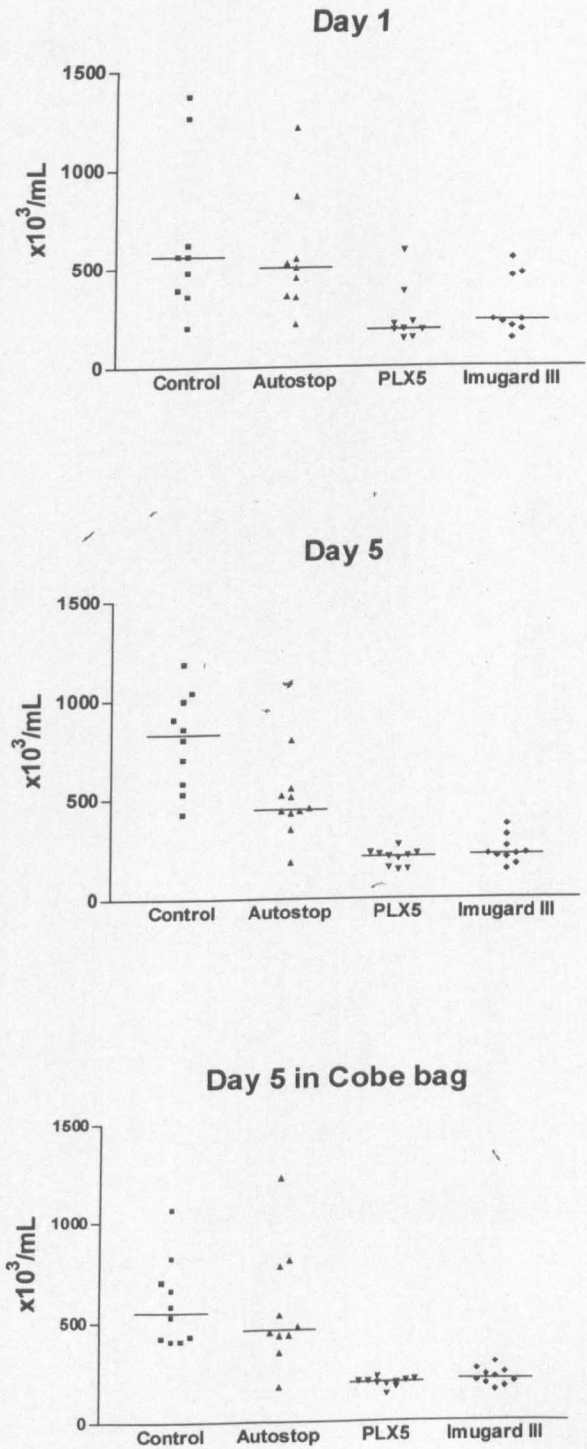


Figure 4.7 Correlation between P-selectin on platelet membrane or soluble P-selectin and Plt-MV on day 5.

Top-P-selectin on platelet membrane vs Plt-MV ($r=0.4215$, $p=0.0081$), bottom soluble P-selectin vs Plt-MV ($r=0.5423$, $p=0.0002$).

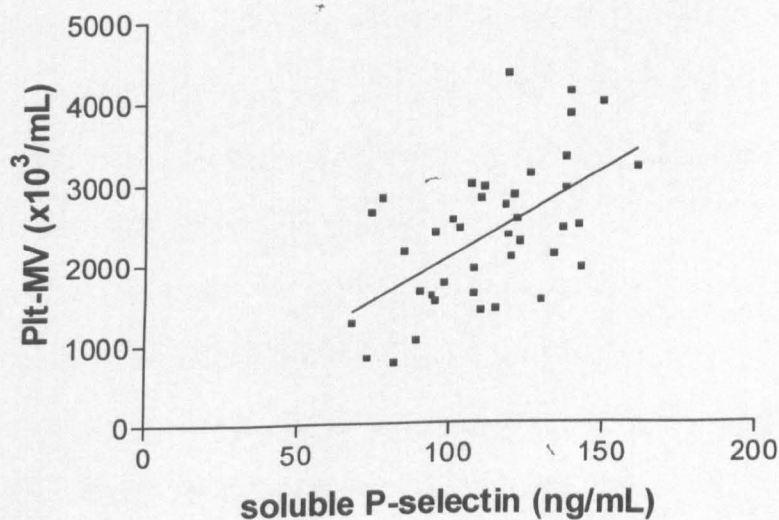
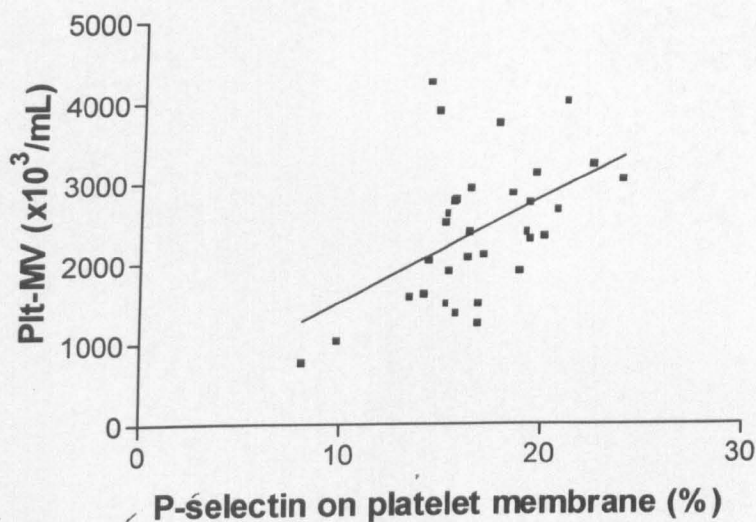
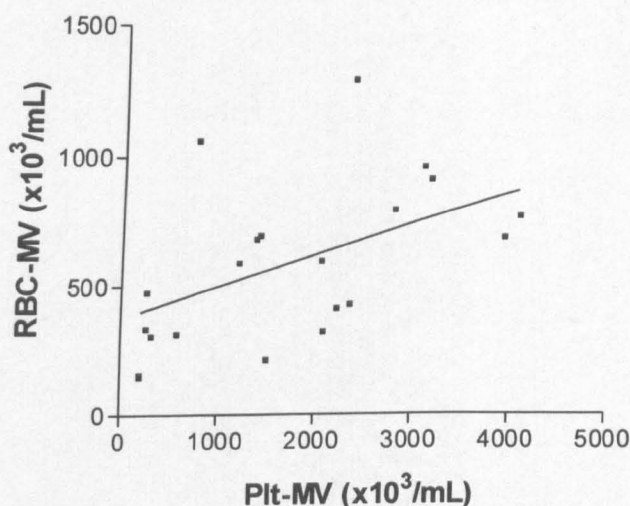


Figure 4.8 Correlation between Plt-MV and RBC-MV in control unfiltered platelets.

$r=0.5714$, $p=0.0042$, pooled data from day 1 and day 5.



4.4 DISCUSSION

All three filters used in this study provided leucodepleted platelets which met the specifications, i.e. platelet yield 240×10^9 /unit, residual leucocyte content less than 5×10^6 /unit, and pH at the end of storage between 6.4 to 7.4 (Guidelines for the Blood Transfusion Services in the United Kingdom 1998).

Although it has been previously reported that leucocyte filtration leads to 10-20% loss of platelets in filtered platelets as compared to unfiltered control (Bertolini 1990), this degree of platelet removal was seen in this study only with the positively charged filter, PLX5. The loss of platelets found in this filter could be due to the charge interaction between the filter and platelets as platelets are known to contain a net negative charge on their surface. The lower platelet loss in this study compared to others may be explained by the study design used by others, in which there was an additional

step involving transfer of platelet rich plasma into a platelet bag prior to filtration. This study, which used the current production method of direct filtration of platelet rich plasma, can allow maximal recovery of platelets without significant loss.

The assessment of platelet activation and quantitation of Plt-MV has been included in the current recommendations for the evaluation of novel platelet concentrates by the American Food and Drug Administration (FDA) (Vostal 1999). Levels of P-selectin on platelet membrane and soluble P-selectin in plasma supernatant of PCs have been shown to be correlated with each other (Divers 1995, Kostelíjk 1996). The results from this study confirm this observation. Soluble P-selectin is more easily standardised and practical and can be performed in a large scale. The results in this study showed that filtration did not have a net effect on the levels of P-selectin. Similar findings have been reported by others (Bertolini 1990, Pedigo 1993, Boomgaard 1995, Metcalfe 1997). This implies that either filtration did not activate platelets and/or activated platelets expressing P-selectin were removed by the filter. Nevertheless, one study has reported that filtration causes platelet activation (Devine 1999). It should be noted that the filtration and platelet preparation process used in this report were greatly different from others. In other reports filtration was carried out after platelets had been pelleted. By contrast, in this report after the first centrifugation of whole blood, platelet rich plasma was passed through a filter. The second centrifugation to pellet platelets was then carried out on the filtered platelet rich plasma using a high centrifugation *g* force. It is possible that subjection of platelets to a high speed centrifugation step shortly after the exposure of platelets to the artificial surface of the filter increases platelet activation.

In respect to microvesiculation, none of the three filters had a net effect on the levels of Plt-MV on day 1. This could be because either filtration did not generate or remove (pre-formed) Plt-MV or filtration both generated and removed Plt-MV resulting

in a balanced net effect. On storage, Imugard III filtered platelets showed the lowest increment in Plt-MV.

Although several factors can influence the levels of platelet activation and microvesiculation, the differences seen in platelets stored in various bags originally supplied with the filters on day 5 were most likely to be related to the types of the bags used. This was because platelets passed over different filters but stored in the same type of bag, i.e. Cobe sample bag, did not show any difference. It has been shown that the inner wall of most plastic bags provides a site for complement fixation that could interact with platelets during storage (Gyongyossy-Issa 1994). In this respect, different types of bags may have different characteristic properties. Furthermore, bags with a larger volume/surface area may enhance the collision between platelets and the bags, resulting in higher degrees of mechanical injury to platelets. Therefore, the lower levels of platelet activation and microvesiculation observed in Imugard III filtered platelets on storage could be partly due to the smaller volume/surface area of the Teruflex bag used with this filter, as compared to other bags. This was substantiated by the fact that the differences in platelet activation and microvesiculation between platelets kept in Teruflex (1.0 L) and PL2410 bags (1.3 L) were less than those kept in CLX bag (1.5 L). It has previously been shown that the reduction in surface area of the storage bag reduces the levels of platelet activation and microvesiculation (Bode 1991).

In designing bags for platelet storage, apart from biocompatibility, oxygen (O₂) and carbon dioxide (CO₂) exchange property is also important. The gas exchange property of the bags depends on both the type of plastic/plasticisers and the total surface area. It should be noted that the bags currently available are not specifically designed for the 5-day storage of leucocyte depleted platelet concentrates and the focus has been placed on maximising O₂ and CO₂ exchange in the presence of higher levels of

leucocytes. Leucocyte depleted products generate less CO₂ during storage due to reduced acid metabolites generated by lower numbers of leucocytes. In addition, leucocyte depleted products also require less O₂ as the O₂ consumption by leucocytes is reduced. When O₂ from the atmosphere continues to diffuse into the bag and exceeds the demand, pO₂ and pH increase (Dzik 1992). The increase in pH on storage was also observed in this study. A good balance between the elimination of CO₂ and the supplementation of O₂ is required for optimised storage conditions. While high CO₂ leads to less effective buffering by bicarbonate, resulting in a fall in pH and platelet activation, excess O₂ may lead to peroxidation of membrane lipids, resulting in microvesiculation (Koerner 1992).

Both PLX5 and Imugard III reduced pre-formed RBC-MV on day 1. On day 5 RBC-MV in all three filtered were unchanged whereas the levels in unfiltered control increased. These could be explained by that the leucocytes in unfiltered platelets caused further microvesiculation of the intact RBC present in the products. The effect of leucocytes on the integrity of red cell membrane has been previously demonstrated in red cell concentrates (Heaton 1994). By contrast, for platelet microvesiculation, the effects of other factors such as the type of plastic/plasticiser and surface area/volume of storage bags, the interaction of plasma proteins with the filters, might be more pronounced. An earlier report has shown that the presence of leucocyte in platelet concentrates did not grossly influence the platelet storage lesion as compared to non-leucocyte depleted control stored in the same type of storage bag (Dzik 1992).

In conclusion leucocyte filtration did not generate or remove Plt-MV. Upon 5-day storage platelets which had passed through different filters showed different levels of Plt-MV. However, this appeared to be related to the differences in the storage bags used in various filters, as when platelets from various filters were kept in the same type of bag the results became similar.

CHAPTER 5

EFFECTS OF PLATELET PREPARATION AND

LEUCOCYTE DEPLETION METHODS

ON MICROVESICULATION

5.1 INTRODUCTION

Approximately 40% of platelet concentrates transfused are provided from apheresis. In apheresis method, after platelets have been separated from whole blood, other blood elements i.e. red cells, some leucocytes and plasma, are returned to the donor. This strategy provides one or more adult therapeutic doses of platelet concentrates from one donor, hence it reduces the number of donor exposures in multi-transfused patients. In addition, platelet concentrates prepared by apheresis method are the only option to provide a specific phenotype of HLA or Human Platelet Antigen (HPA) match.

In the UK, leucocyte depletion of apheresis platelets are achieved by two techniques i.e. a fluidised particle bed used in Cobe LRS apheresis machine, and leucocyte filtration of the harvested platelets during the last return cycle used in Haemonetics MCS+ LD apheresis machine. The collection sets used in various types of apheresis machine are different from each other, and also different from those used in pooled buffy coat method. These collection sets are made from various materials and have different configuration as described previously in Chapter 1. It is not known whether all these combined variables will have similar effects on platelet activation and microvesicle formation. Furthermore, leucocyte depleted platelets prepared by apheresis

methods may differ from those prepared by the pooled buffy coat method.

The aim of this study was to investigate whether leucocyte depleted platelet concentrates prepared by three different methods, i.e. Cobe LRS apheresis, MCS+ LD apheresis using LRF6H leucocyte removal filter, and pooled buffy coat method using Autostop filter, contained the same level of MV.

5.2 MATERIAL AND METHODS

A paired study design is not possible when apheresis platelets are examined. Equally, it is not possible to compare the same donor bled on two different machines, as donors are frequently only suitable for one type of machine.

Ten units of leucocyte depleted platelet concentrates prepared by each technology, Cobe LRS, Haemonetics MCS+ LD, and Autostop filtered pooled buffy coat method, were analysed. The methods of platelet preparation, the measurement of pH, leucocyte count, platelet count, platelet activation assessed by soluble P-selectin, and microvesicles were described previously in Chapters 1, 2 and 3. For apheresis platelets, samples were tested on the day of production (day 0), day 1, and day 5. For MCS, a pre-filtered sample from each unit was also tested. For Autostop filtered platelets, samples were tested on day 1 and day 5, since in this preparation method platelet concentrates were made from pooled buffy coats on day 1 (described in detail in Chapter 3). The following comparisons were made:

- i. MCS pre- and post-filtration
- ii. Cobe and post-filtered MCS on day 0
- iii. Cobe, post-filtered MCS and Autostop on day 1 and day 5
- iv. The same type of platelets tested at different times

Mann-Whitney test, Wilcoxon matched pairs test, and ANOVA test were used to compare between unpaired, paired, and more than two sets of results, respectively. Spearman test was used for the correlation between results.

5.3 RESULTS

5.3.1 pH, leucocyte and platelet counts

There was no difference in pH between Cobe and MCS platelets on day 0, and no difference between Cobe, MCS, and Autostop on day 1. However, on day 5 both Cobe ($p<0.001$) and MCS ($p<0.01$) were lower than Autostop (Table 5.1). All units were within the acceptable range (6.4-7.4) (Guidelines for the Blood Transfusion Services in the United Kingdom 1998). Residual leucocyte counts were less than $5 \times 10^6/\text{unit}$ in all products. The platelet count in Cobe was higher than MCS ($p<0.01$) and Autostop ($p<0.001$). (Figure 5.1).

5.3.2 Platelet activation as assessed by soluble P-selectin

Pre-filtered MCS platelets contained higher levels of soluble P-selectin than post-filtered ($p=0.0315$) implying that the filter used (LRF6H) absorbed some soluble P-selectin (Figure 5.2). Post-filtered MCS PC were equivalent to Cobe PC on day 0. On day 1, the levels were not significantly different from day 0 and all leucocyte depleted products were equivalent. On day 5, all groups showed increases levels of soluble P-selectin as compared to day 1 (all $p<0.01$). Cobe ($p<0.05$) and Autostop ($p<0.01$) were significantly higher than MCS (Figure 5.3). No correlation was found between platelet count and soluble P-selectin.

5.3.3 Microvesicles

The levels of Plt-MV in MCS pre-filtered samples were lower than post filtered

($p=0.0020$), whereas RBC-MV were equivalent (Figure 5.4). The Plt-MV of post filtered MCS were equivalent to Cobe on day 0. On day 1, no significant difference was found in both products as compared to day 0. Furthermore, post-filtered MCS, Cobe, and Autostop showed similar levels. On day 5, Plt-MV were significantly increased in all products as compared to day 1 ($p<0.01$ in all groups). Furthermore, MCS showed lower levels than Cobe ($p<0.01$) and Autostop ($p<0.01$). (Figure 5.5).

The levels of RBC-MV were significantly lower in post-filtered MCS than in Cobe ($p=0.0021$) on day 0. During storage, RBC-MV remained unchanged. Cobe ($p<0.05$) and Autostop ($p<0.001$) were higher than MCS on both day 1 and day 5 (Figure 5.6).

No correlation was found between platelet count and Plt-MV on day 1 or day 5. In addition, no correlation was found between the levels of Plt-MV on day 1 and day 5.

There was a highly significant correlation between Plt-MV and soluble P-selectin on day 5 ($r=0.7155$, $p<0.0001$, all groups pooled) (Figure 5.7) but not on day 0 or day 1. No correlation was found between Plt-MV and RBC-MV.

Table 5.1 pH of Cobe, MCS, and Autostop on day 0, day 1, and day 5.

Median (range). On day 5 both Cobe ($p<0.001$) and MCS ($p<0.01$) were lower than Autostop.

Days	Cobe	MCS	Autostop
0	7.06 (7.02-7.10)	7.10 (7.00-7.22)	NA
1	7.06 (6.93-7.30)	7.16 (7.00-7.25)	7.10 (7.05-7.15)
5	7.16 (7.03-7.24)	7.19 (6.95-7.26)	7.26 (7.24-7.31)

NA-not applicable

Figure 5.1 Platelet counts of Cobe, MCS, and Autostop.

— Median. Cobe was higher than MCS ($p<0.01$) and Autostop ($p<0.001$).

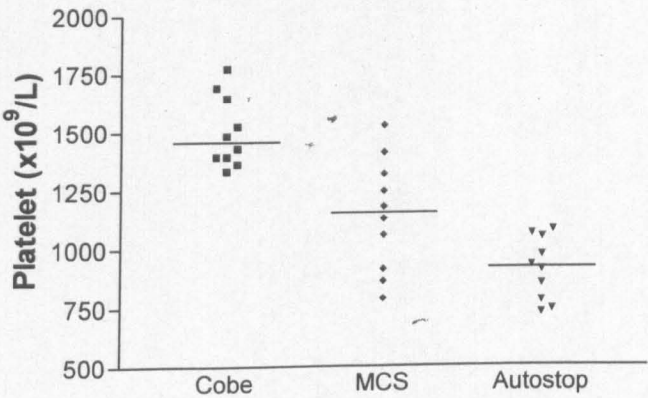


Figure 5.2 **P-selectin in the supernatant plasma of pre- and post-filtered MCS platelets.**

_____ Median. Post-filtered was significantly lower than pre-filtered ($p=0.0315$).

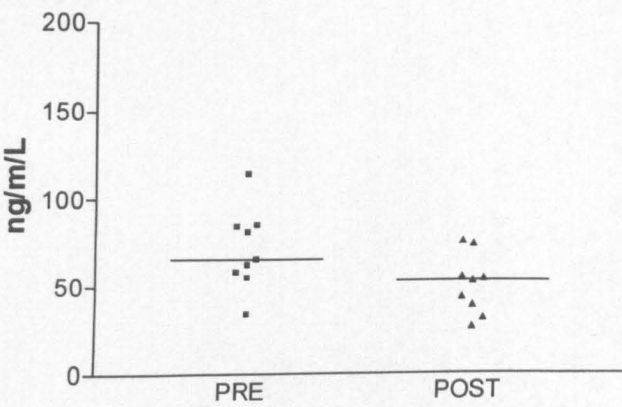


Figure 5.3 Soluble P-selectin of Cobe, post-filtered MCS and Autostop on day 0, day 1, and day 5.

_____ Median. Top-day 0, middle-day 1, bottom-day5. Cobe and Autostop were significantly higher than MCS on day 5 ($p<0.01$).

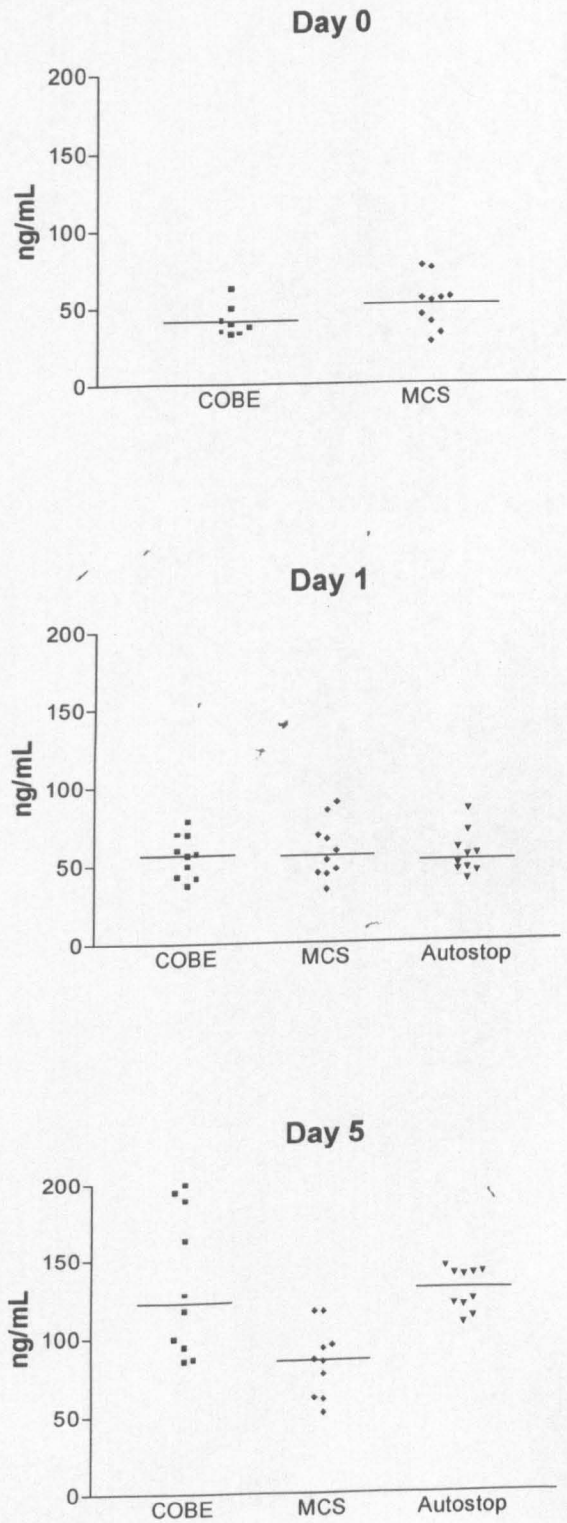


Figure 5.4 Plt-MV and RBC-MV of pre- and post-filtered MCS platelets.

_____ Median, top-Plt-MV, bottom-RBC-MV. Plt-MV in pre was significantly lower than post ($p=0.0020$).

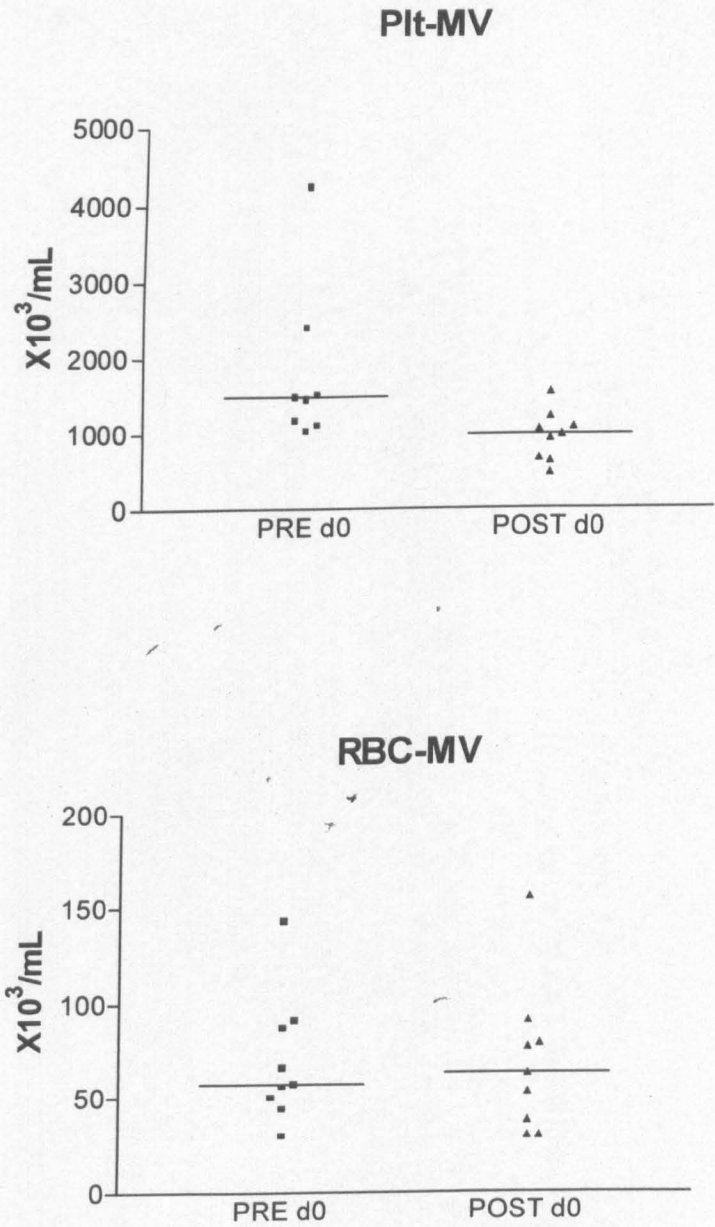


Figure 5.5 Plt-MV of Cobe, post-filtered MCS and Autostop on day 0, day 1 and day 5.

_____ Median, top-day 0, middle-day 1, bottom-day 5. On day 5 Cobe ($p<0.01$) and Autostop ($p<0.01$) were significantly higher than MCS.

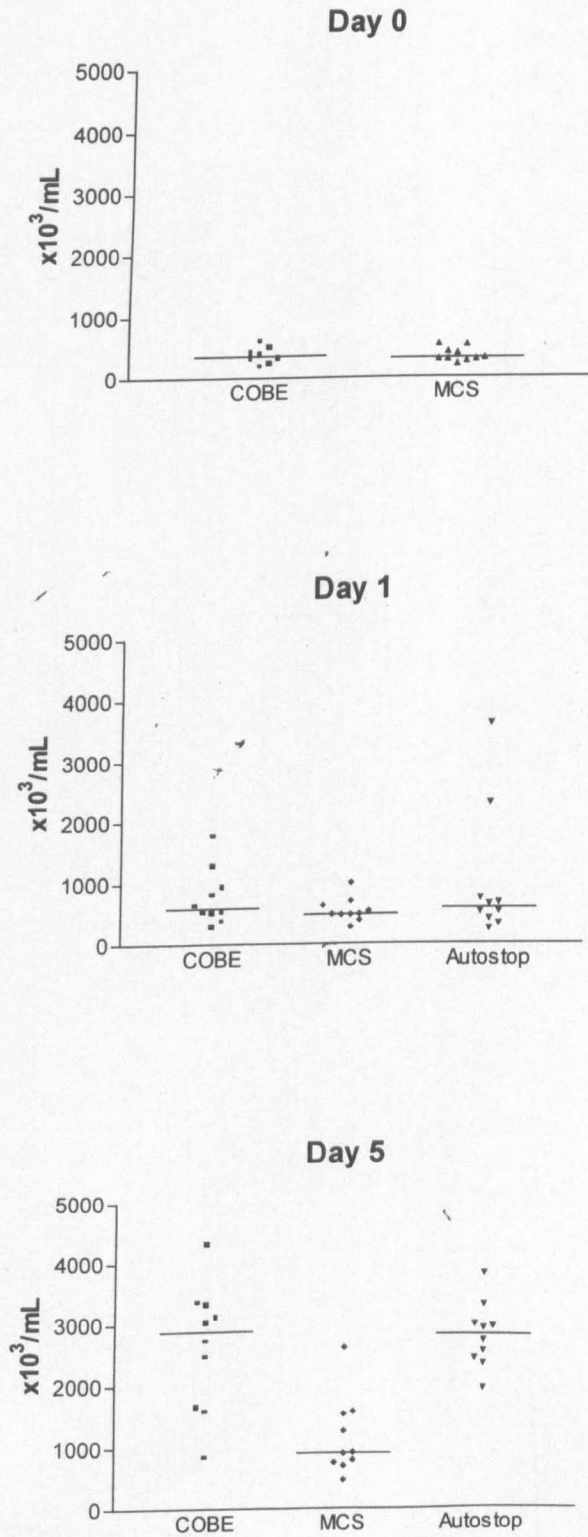


Figure 5.6 RBC-MV of Cobe, post-filtered MCS and Autostop on day 0, day 1, and day 5.

— Median, top-day 0, middle-day 1, bottom-day 5. On day 0 Cobe was significantly higher than MCS ($p=0.0021$). On day 1 and day 5, both Cobe ($p<0.05$) and Autostop ($p<0.001$) were higher than MCS.

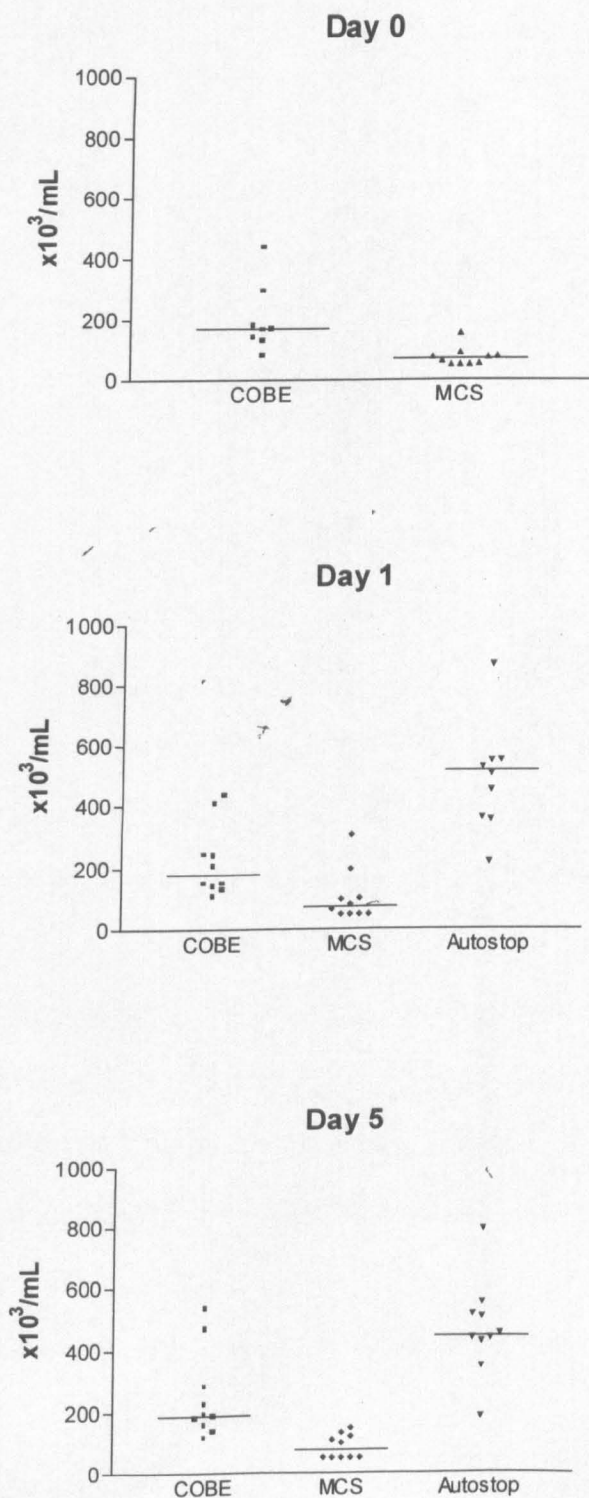
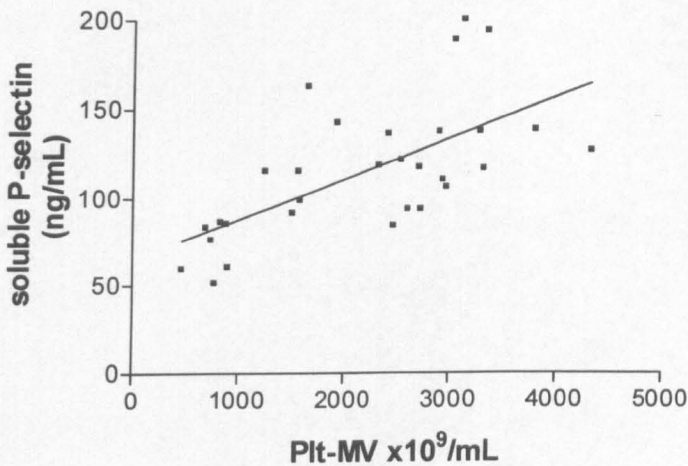


Figure 5.7 Correlation between Plt-MV and soluble P-selectin on day 5.

$r=0.7155, p<0.0001$. Cobe, MCS and Autostop pooled.



5.4 DISCUSSION

The leucocyte removal filter (LRF6H) used in the preparation of platelet concentrates by MCS removed both soluble P-selectin and Plt-MV. An earlier report on MCS platelets using the same filter did not find any difference in P-selectin expression on platelet membrane between before and after filtration (Holme 1997). This might be because different filtration processes were used. In this study, filtration was carried out during the last return cycle, at the end of platelet collection, whereas in the earlier report a slow continuous filtration was carried out from the beginning through the end of platelet collection. It might be possible that in the latter process soluble P-selectin was absorbed and then later eluted into the filtrate, hence the levels of pre- and post-filtered products were unchanged.

While no difference in the levels of soluble P-selectin and Plt-MV was seen

between Cobe and MCS on day 0 or between Cobe, MCS and Autostop on day 1, on day 5 both Cobe and Autostop were higher than MCS. The higher levels of P-selectin and Plt-MV in Cobe could not be explained by the higher platelet count as there was no correlation between platelet count and the two parameters. However, several other variables could be responsible for the higher levels of soluble P-selectin and Plt-MV seen in Cobe on day 5. These include the differences in centrifugation g force used to separate platelets from red cells, the design of the collection units, the artificial surface of the harness and storage bags, as well as the leucocyte depletion process. All these variables may lead to the recovery of different subpopulations of platelets, undergoing various degrees of activation/microvesiculation. This hypothesis was supported by the results in Chapter 3 showing the difference in their response to EDTA between the three types of platelets.

A previous report on the comparison of platelet activation and microvesiculation in platelet concentrates prepared by apheresis technique and from units of whole blood using the platelet rich plasma method has shown that the latter express higher levels of platelet activation and microvesiculation (Sloand 1996). The authors attributed this finding to the type of anticoagulant and the way in which it was mixed with whole blood before the process of platelet separation. In apheresis technique, whole blood was mixed with acid citrate dextrose (ACD) in a uniform way, whereas in platelet rich plasma method, blood flowed into a measured volume of citrate phosphate dextrose adenine (CPDA). Consequently, in the latter method blood obtained early in the donation was transiently subjected to high concentrations of citrate. In this study, although whole blood units used for the preparation of platelets by pooled buffy coat method were collected in the same way as those used in the platelet rich plasma method, the effect of anticoagulant on platelet activation and microvesiculation had not been demonstrated. Nevertheless, it

should be noted that platelets prepared by platelet rich plasma method causes higher degree of platelet activation than buffy coat method due to the second centrifugation step in which platelets were sedimented on the bottom of plastic bag (Fijnheer 1990). This could be accountable for the higher degree of platelet activation seen in platelet rich plasma method as compared to apheresis.

The result on Plt-MV reported by Sloand et al was expressed as percentage of total CD61 (GPIIb/IIIa) positive events, therefore, it cannot be compared with the quantitative result shown in this study. Furthermore, the Plt-MV region setting was not fully described.

The LRF6H filter used in MCS apheresis reduced Plt-MV but not RBC-MV, whereas the Autostop filter used for leucodepletion of platelet concentrates derived from pooled buffy coats did not affect MV levels as compared to non-filtered control (Chapter 4). This could be related to the difference in the internal structure of the filters although both filters are made from negatively charged polyester fibres. Nevertheless, among the three platelet preparation/leucocyte depletion methods, MCS gave the lowest level of RBC-MV. This could be explained by the difference in the process of platelet collection. As the specific gravity of RBC-MV is relatively higher than platelets, after the centrifugation to separate platelet from red cells, RBC-MV are settled between the interface of plasma containing platelets and red cell layer. In the MCS apheresis machine, platelet collection in each cycle was terminated at the level far from this interface, and hence leaving most of RBC-MV behind. In the Cobe LRS apheresis machine, the platelets were collected until the interface with red cells was reached. In Autostop filtered platelets, the collection of platelets was stopped when a mass of red cells started to enter the filter, hence more RBC-MV were collected as compared to MCS and Cobe.

The correlation between Plt-MV and platelet activation assessed by soluble P-

selectin seen at the end of shelf-life but not at the beginning implied that platelet activation was one of the mechanisms of microvesicle formation during storage. However, at the beginning of shelf-life the influence of other variables such as shear stress caused by centrifugation and the interaction with the artificial surface of collection set, on platelet microvesiculation are more pronounced than platelet activation.

In summary Cobe LRS, MCS+ LD apheresis technologies, and Autostop filtered platelets did not show gross differences in terms of platelet activation and microvesiculation on day 1, but upon 5-day storage Cobe LRS showed higher levels of platelet activation and microvesiculation than the other two methods.

CHAPTER 6

EFFECT OF LEUCODEPLETION ON THE ACTIVATION OF COAGULATION AND COMPLEMENT SYSTEMS

6.1 INTRODUCTION

While citrate anticoagulant used in the preparation of platelet concentrates sufficiently chelates calcium ions to prevent clotting, activation of coagulation and complement systems still proceeds as demonstrated by the generation of thrombin, plasmin, and activated complement components such as C3a, C5a, C5b-9 (Hesseltvik 1989, Bode 1989, 1992, Miletic 1993, Gyongyoss-Issa 1994, Krailadsiri 1996). The type and ratio of anticoagulant used, various leucocyte removal filters and different artificial surfaces of apheresis harness, may affect the coagulation and complement systems to different degrees. These may also contribute to platelet activation and microvesiculation.

The objectives of this study were:

- i. To investigate whether the three commonly used leucocyte removal filters for pooled buffy coat derived platelets concentrates i.e. negatively charged polyester (Autostop), positively charged polyester (PLX5), and neutrally charged polyurethane (Imugard III), activate the coagulation and complement systems.
- ii. To investigate whether different leucocyte depletion technologies currently in use in routine production i.e. filtration of pooled buffy coat derived platelets using Autostop filter, filtration of apheresis platelets (MCS+ LD) using LRF6H filter, and leucocyte depletion by a fluidised particle bed (Cobe LRS), have the same effects on the coagulation and complement systems.

iii. To investigate whether the activation of coagulation/complement systems correlates with platelet activation/microvesiculation.

6.2 MATERIAL AND METHODS

Ten sets of pooled buffy coat derived platelet concentrates (BC-PCs) were prepared. Each set consisted of 4 units of PCs made from the same pool of buffy coats. One unit served as a non-filtered control, and each of the rest three was filtered with a negatively charged polyester (Autostop) or a positively charged polyester (PLX5) or a neutrally charged polyurethane (Imugard III). Ten units of Cobe LRS and MCS+LD apheresis PCs were also analysed. The detail on platelet preparations was described in Chapters 4 and 5. A sample from each unit was taken on day 1 and day 5.

The activation of contact system was measured by FXIIa using ELISA, and by a microtitreplate chromogenic assay using a synthetic substrate sensitive to kallikrein (S2302), so called kallikrein-like activity (KLA). The generation of thrombin was measured by prothrombin fragment 1+2 (F1+2), thrombin-antithrombin complex (TAT) using ELISA, and by chromogenic assay using a synthetic substrate sensitive to thrombin (S2238), so called thrombin-like activity (TLA). The activation of complement system was measured by C3a using ELISA. All assays were performed on the supernatant plasma on day 1 and day 5 following the methods described in Chapter 2.

Results between the control and three filtered BC-PCs, and between Autostop filtered BC-PCs and the two apheresis PCs were compared using the Friedman and Kruskal-Wallis ANOVA tests, respectively. Results between day 1 and day 5 of the same products were compared using the Wilcoxon matched pairs test. The Spearman test was used to analyse the correlation between two sets of results.

6.3 RESULTS

6.3.1 Comparison between unfiltered control and three filtered BC-PCs

i. KLA and TLA

On day 1 KLA and TLA were comparable in control, PLX5 and Imugard III, but over 2-fold higher in Autostop filtered PCs compared to the other three groups (all $p < 0.0001$). On day 5 KLA in control, PLX5 and Imugard III filtered PCs were unchanged from day 1. Autostop values had slightly decreased (not significant) from day 1 but were still significantly higher than other groups (all $p < 0.0001$). TLA remained constant in all products during storage (Figure 6.1). Significant correlation was found between KLA and TLA both on day 1 and day 5 (day 1 $r=0.7489$, $p<0.001$, day 5, $r=0.6793$, $p<0.0001$, all four groups pooled) (Figure 6.2).

ii. C3a

On day 1 Autostop showed the lowest C3a ($p<0.01$, 0.001 , 0.01 for Autostop vs control, PLX5, and Imugard III, respectively), whereas PLX5 showed the highest (all $p<0.001$). These indicated that the negatively charged filter absorbed C3a, whereas the positively charged filter generated it. Imugard III, a neutrally charged filter, was not different from control.

On day 5, the levels of C3a in all groups increased by approximately 3 fold from day 1, with Imugard III being the lowest. Significant differences were seen only between Imugard III and Autostop ($p<0.001$), and between Imugard III and PLX5 ($p<0.01$). (Figure 6.3)

6.3.2 Comparison between Cobe LRS, MCS+ LD apheresis PCs, and Autostop filtered BC-PCs

i. KLA and TLA

On day 1, Cobe showed the lowest level of KLA ($p<0.05$ and 0.001 for Cobe vs MCS and vs Autostop, respectively) and TLA ($p<0.05$ and 0.01 for Cobe vs MCS and vs Autostop, respectively). No significant difference was found between MCS and Autostop. On day 5, the levels of both KLA and TLA in Cobe and Autostop remained similar to the levels on day 1, whereas MCS significantly increased (day 1 vs day 5, KLA $p=0.0037$, TLA $p<0.0001$). The pattern of KLA on day 5 between the three groups remained similar to that on day 1 i.e. Cobe was the lowest (Cobe vs MCS $p<0.01$, Cobe vs Autostop $p<0.001$, no difference between MCS and Autostop). However, the pattern of TLA was different from day 1, with MCS showing the highest level (MCS vs Cobe $p<0.001$, MCS vs Autostop $p<0.05$). Cobe and Autostop did not show any significant difference. (Figure 6.4)

The degree of correlation between KLA and TLA was higher on day 1 ($r=0.6854$, $p<0.0001$) than day 5 ($r=0.5903$, $p=0.0003$) (Figure 6.5). This could be because during storage thrombin bound to the surface of platelet membrane as well as the storage bag.

ii. C3a

On day 1, both Cobe ($p<0.001$) and MCS ($p<0.01$) showed higher C3a than Autostop BC-PCs. On day 5, no significant difference was observed between the three groups (Figure 6.6).

6.3.3 FXIIa, F1+2 and TAT

The levels of FXIIa, F1+2 and TAT in control and all types of leucodepleted PCs were at the basal levels, within the plasma normal ranges (FXIIa <2.9 ng/mL, F1+2 <1.1 nM, TAT <4 μ g/L). No significant difference was found between control and leucocyte depleted products or between day 1 and day 5 of the same group.

6.3.4 Correlation between KLA, TLA, C3a and platelet activation/microvesiculation

Only weak correlations were seen between TLA and Plt-MV on day 5 (all BC-PCs pooled $r=0.3833$, $p=0.0146$, both apheresis PCs pooled $r=0.4874$, $p=0.0393$). No correlation was seen between KLA, C3a and Plt-MV, or between KLA, TLA, C3a and platelet activation measured by soluble P-selectin.

Figure 6.1 KLA and TLA in control and three types of filtered BC-PCs on day 1 and day 5.

— Median. Left panels-KLA, right-TLA, top-day1, bottom-day 5. The levels of KLA and TLA in Autostop were significantly higher than other three groups (all $p<0.0001$) on both day 1 and day 5.

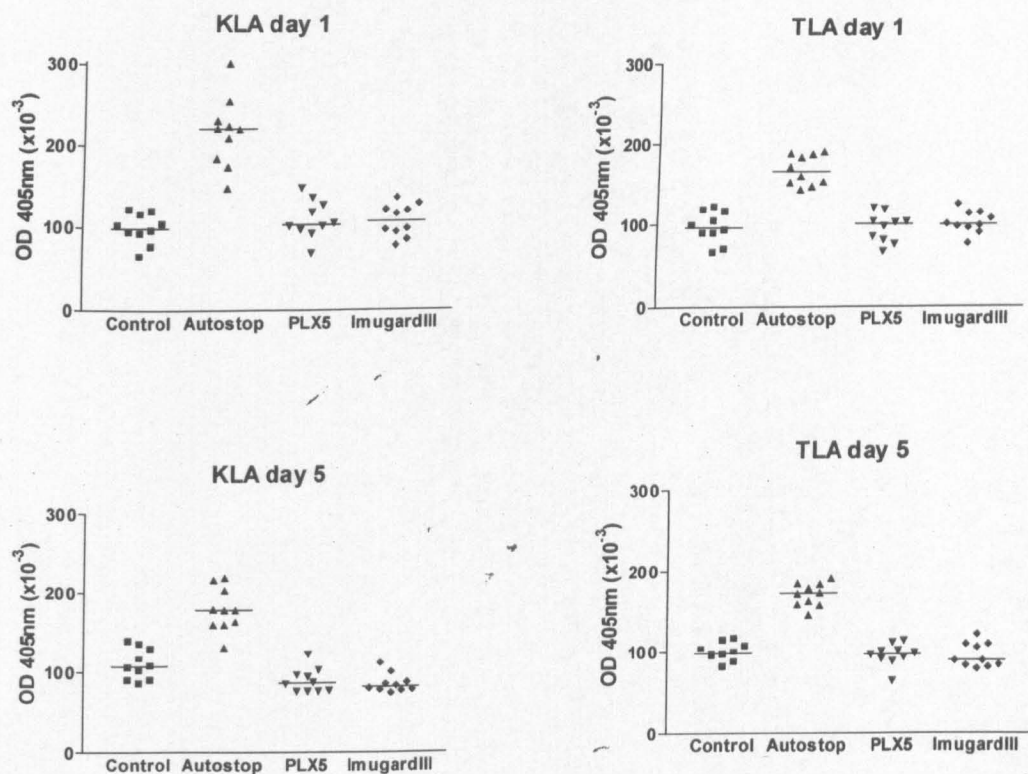


Figure 6.2 Correlation between KLA and TLA of BC-PCs on day 1.

$r=0.7489$, $p<0.001$, all four groups pooled.

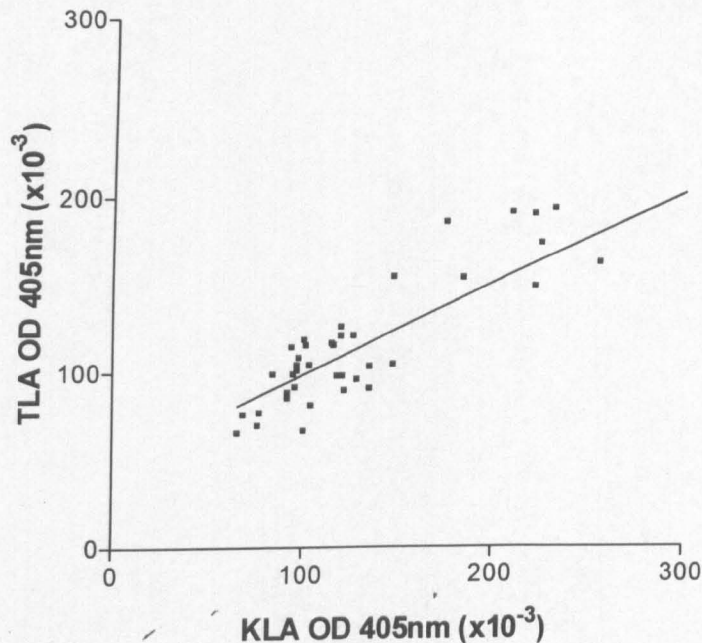


Figure 6.3 C3a in control and three filtered BC-PCs on day 1 and day 5.

_____ Median. Top panels-day1, bottom day5. On day 1 Autostop was the lowest ($p<0.01$, 0.001, 0.01 for Autostop vs control, PLX5, and Imugard III, respectively), whereas PLX5 the highest (all $p<0.001$). On day 5, Imugard III was significant lower than Autostop ($p<0.001$) and PLX5 ($p<0.01$).

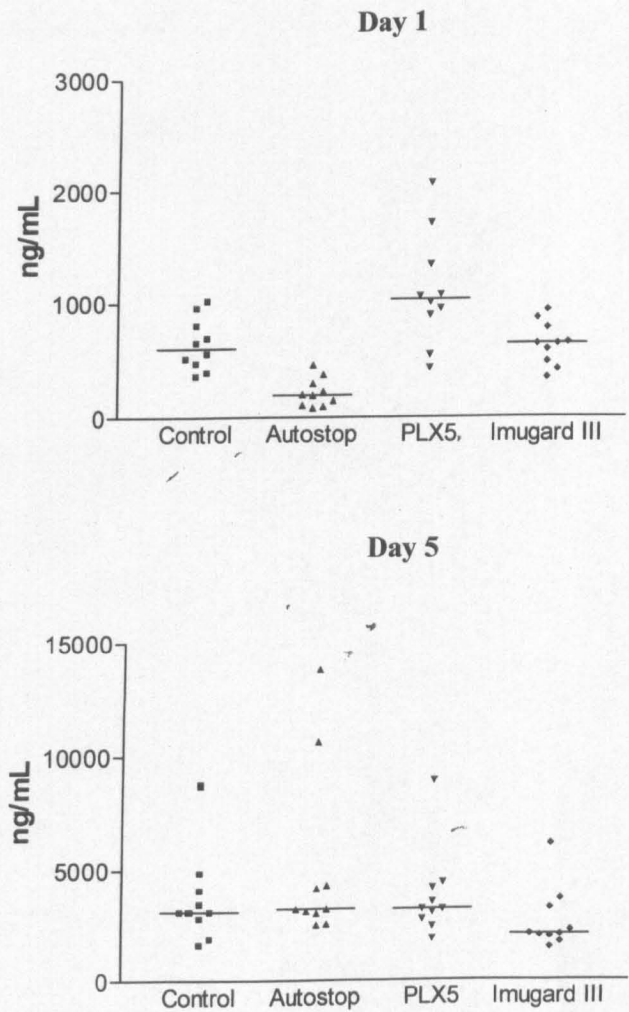


Figure 6.4 The levels of KLA and TLA in Cobe, MCS, and Autostop filtered PCs on day 1 and day 5.

— Median. Left panels-KLA, right-TLA, top-day1, bottom-day 5. On day 1, Cobe showed the lowest levels of both KLA ($p<0.05$ and 0.001 for Cobe vs MCS and vs Autostop, respectively) and TLA ($p<0.05$ and 0.01 for Cobe vs MCS and vs Autostop, respectively). On day 5, Cobe showed the lowest KLA (Cobe vs MCS $p<0.01$, Cobe vs Autostop $p<0.001$), MCS showed the highest TLA (MCS vs Cobe $p<0.001$, MCS vs Autostop $p<0.05$).

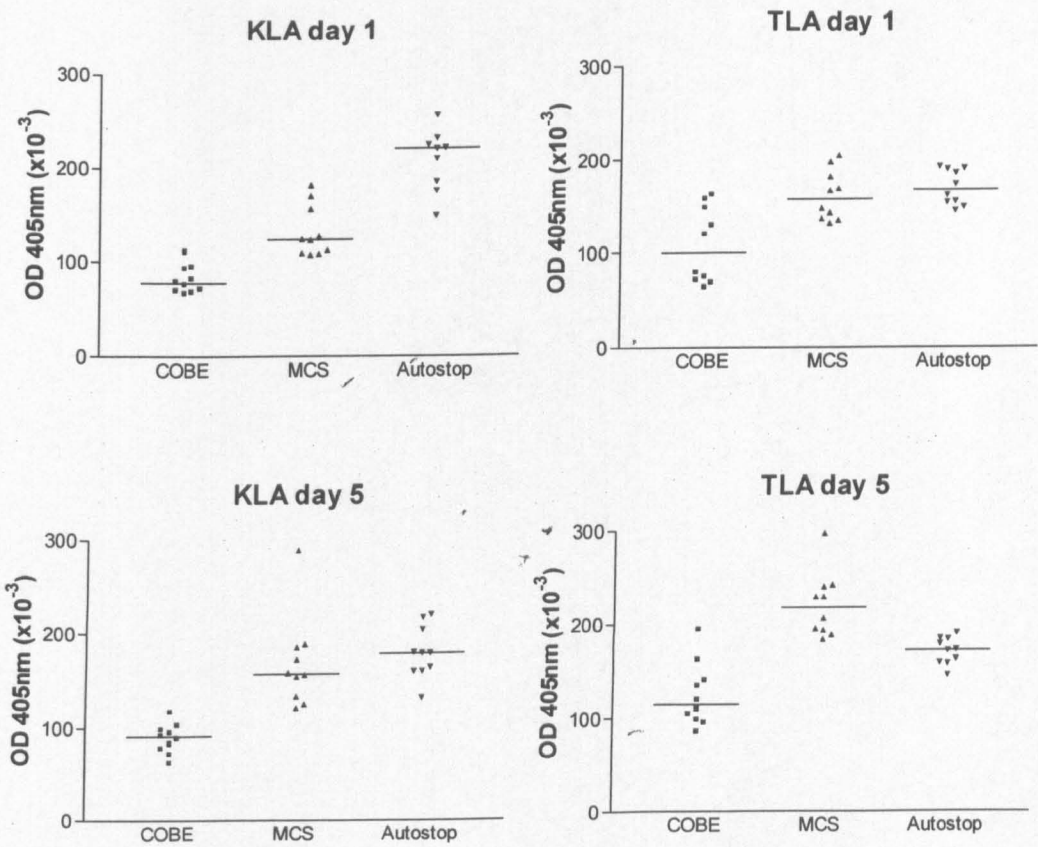


Figure 6.5 Correlation between KLA and TLA on day1.

$r=0.6854, p<0.0001$. Cobe, MCS and Autostop pooled,

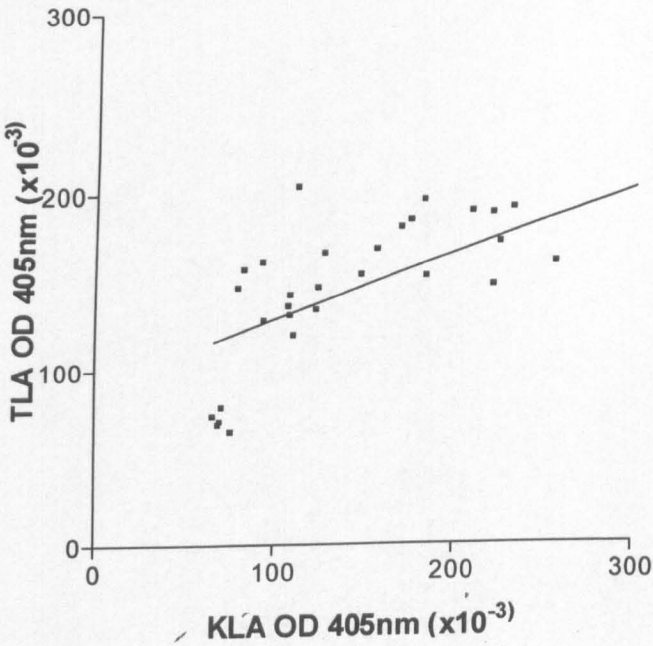
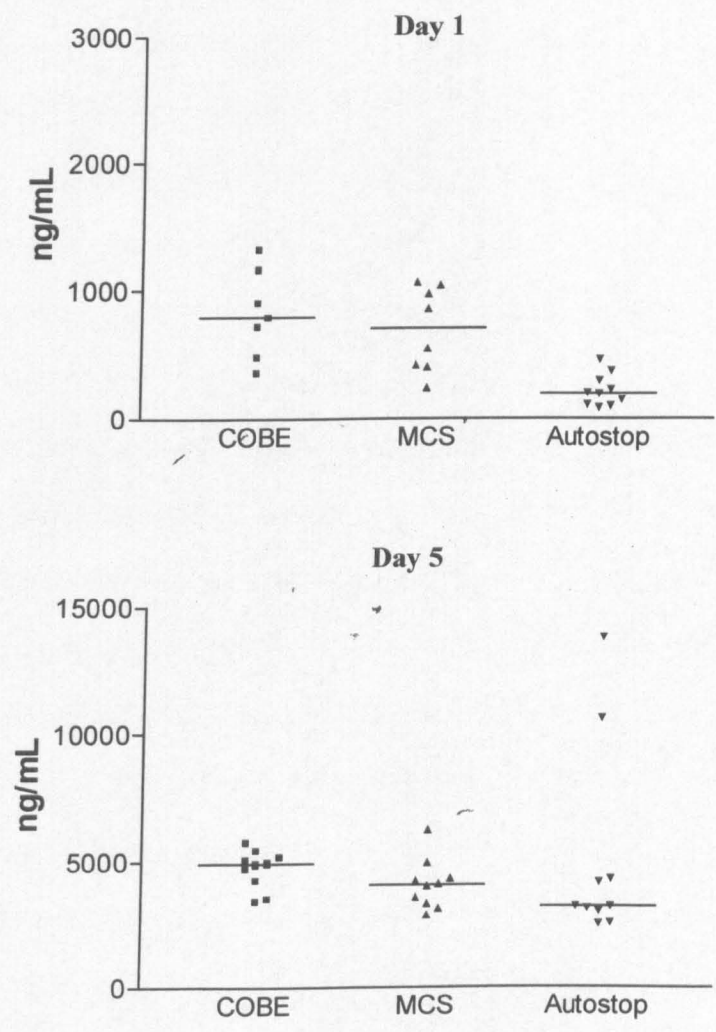


Figure 6.6 C3a in Cobe, MCS, and Autostop filtered PCs on day 1 and day 5.

— Median. Top-day 1, bottom-day 5. On day 1, both Cobe ($p<0.001$) and MCS ($p<0.01$) were higher than Autostop BC-PCs. On day 5, no significant difference was observed.



6.4 DISCUSSION

Leucocyte depleted platelet concentrates prepared by various filters and/or various methods were different from each other in terms of the activation of coagulation and complement systems. Autostop filtered BC-PCs showed higher levels of KLA than control or the other two filtered BC-PCs, possibly due to the activation of the contact system by the negatively charged surface of this filter. MCS apheresis PCs which used another negatively charged polyester filter also showed higher KLA than Cobe apheresis PCs. It has been reported that the activation of the contact system by a negatively charged leucocyte removal filter for PC leads to the generation of bradykinin, and is associated with hypotensive reactions in transfused patients also receiving angiotensin converting enzyme inhibitors (Fried 1996, Hume 1996, Sano 1996, Mair 1998). These reactions were previously described to be associated with the use of bed-side filters only, and not pre-storage in-process filters due to the short half-life of bradykinin. However, in the recent Serious Hazards Of Transfusion Report, three patients receiving pre-storage filtered PCs developed hypotensive reactions (Williamson 1999). It is not known whether these were associated with high levels of bradykinin.

The level of FXIIa antigen did not increase in parallel to the increase in KLA, indicating that FXIIa antigen alone is not a good marker of the activation of contact system in platelet concentrates. This could be due to its high affinity binding to surfaces i.e. plastic bags, various plasma protease inhibitors such as C1 inhibitor, α 2-macroglobulin, antithrombin III, as well as protease inhibitors released from platelets such as platelet plasminogen activator inhibitor-1.

Previous reports measured the activation of contact system caused by negatively charged filters by detecting bradykinin (Shiba 1997, Hild 1998) or high molecular weight

kininogen index (HKI) (Scott 1998). Both assays have some limitations. Bradykinin contains an extremely short half-life (15 seconds) and required protease inhibitors to be added to the samples immediately after filtration (Shiba 1997). This prevents the handling of samples in a closed system where the same sample can be kept for further study on storage. HKI, measuring the ratio of high molecular weight kininogen before and after the generation of bradykinin, is not sensitive enough to detect the contact activation caused by negatively charged filters as only 0.2% of total kininogen is converted to bradykinin (Scott 1998).

In parallel to KLA, TLA was increased in Autostop BC and MCS PCs. However, no elevation in the levels of TAT or F1+2 was observed possibly because these ELISA assays were not sensitive enough to detect the low levels of serine proteases generated. Synthetic substrates are more sensitive than a corresponding natural substrate and can detect both free serine proteases and proteases which binds to various inhibitors such as C1-esterase, α 2-macroglobulin, whereas ELISA assay did not. Similar finding where amidolytic activity in the supernatant plasma of PCs was observed using S2238, without elevated TAT, was previously reported (Wallvik 1992).

While KLA and TLA in all BC-PCs and Cobe PCs on day 5 remained similar to the results obtained on day 1, the levels in MCS increased. This is possibly due to the difference in the type and ratio of anticoagulants used i.e. citrate-phosphate-dextrose (CPD) 1:7 for BC-PCs, acid-citrate-dextrose formula A (ACD-A) using a protocol which the ratio ramping from 1:7 to 1:11 during a collection for Cobe, and ACD-A 1:9 for MCS, hence a higher calcium ion concentration in the MCS.

The measurement of KLA and TLA provides a useful indicator of the activation of contact system and the generation of thrombin.

With respect to the activation of complement system, all BC-PCs showed

increased levels of C3a on day 1 as compared to the normal plasma levels (26-146 ng/mL). A previous study has demonstrated that the activation occurs from the moment that whole blood contacts the collection tubing and the initial collection bag (Gyongyossy-Issa 1994). Various filters used for BC-PCs interact with the complement system differently i.e. negative charged filter removed C3a, positively charged filter generated C3a, and neutrally charged filter was the same as control. The removal of C3a, a positively charged protein, by the negatively charged filter is most likely due to an ionic interaction. Previous reports have shown that other positively charged proteins such as C4a, platelet factor 4, β -thromboglobulin, interleukin-8 are also adsorbed by negatively charged filters (Shimizu 1994, 1998, Snyder 1996, Geiger 1997). The levels of C3a were further increased during storage with all products except Imugard III, reaching the same level. The lowest level of C3a in Imugard filtered PCs was seen on storage. This could be due to that the bag used with this filter might be more biocompatible than the bags used with the other two filters and control, and hence generated less C3a, or more C3a might be absorbed on the surface of this bag. Both leucocyte depleted apheresis PCs showed higher degrees of complement activation than Autostop filtered BC-PCs on day 1. This may be explained by the fact that in apheresis techniques blood is in contact with more surfaces of the collection sets as compared to BC-PCs. Nevertheless, on storage the levels of C3a in both apheresis PCs and Autostop BC-PCs increased to the same level.

The mechanism of complement activation in platelet concentrates is not clearly defined. However, it has been proposed that complement activation occurs primarily via the classical pathway through the direct activation of C1 by artificial surfaces, and that the alternative pathway has little role, as activated factor B does not increase (Gyongyossy-Issa 1994).

It has been proposed that the activation of coagulation and complement systems

leading to the generation of thrombin and the membrane attack complex C5b-9 can cause platelet activation and microvesiculation as demonstrated by in vitro experiments (Sims 1988, Bode 1989). However, no study has been carried out to show their correlation. In this study, a trend between KLA/TLA and platelet activation/microvesiculation on day 5 was observed i.e. among the 4 groups of BC-PCs Autostop filtered showed the highest KLA/TLA and the highest P-selectin and Plt-MV. However, only weak correlations were found. This is possibly due to that the generation of serine proteases is not the sole factor responsible for platelet activation and microvesiculation. The mechanisms involved in platelet activation and microvesiculation during the preparation and the storage of platelet concentrates are complex and multifactorial. Apart from the generated serine proteases and activated complement, these also include shear stress caused by centrifugation, filtration, and agitation; the interaction between platelet membranes and the leached material from the bag, platelet ageing, and apoptosis.

In summary only the negatively charged filter activated the coagulation system as measured by chromogenic substrate assay. However, this filter removed some activated complement component C3a as compared to unfiltered control, whereas the positively charged filter generated C3a. The neutrally charged filter did not have significant effects on both the coagulation and complement systems. Both Cobe and Haemonetics apheresis appeared to activate the complement system more than buffy coat method. No correlation was found between the serine proteases or activated complement component and platelet microvesiculation. The clinical significance of the serine proteases detectable by only chromogenic substrate assay but not by conventional ELISA, as well as and activated complement components in platelet concentrates is unknown. Nevertheless, activated complement components such as C3a have pro-inflammatory effects and may cause transfusion reactions.

**THE PROCOAGULANT AND ANTICOAGULANT
PROPERTIES OF MICROVESICLES IN
PLATELET CONCENTRATES**

7.1 INTRODUCTION

Anionic phospholipids on the surfaces of MV and activated platelets play essential roles in two sequential reactions of the coagulation cascade, the formation of tenase and prothrombinase complexes, which lead to thrombin formation, the key step in the coagulation process (Mann 1990). The formation of tenase involves an active proteolytic enzyme, FIXa, which in the presence of a co-factor FVIIIa, Ca^{2+} , and anionic phospholipid membrane, converts the zymogen FX into an active serine protease, FXa. Once FXa is formed, it assembles with a co-factor FVa, and Ca^{2+} , on the anionic phospholipid membrane, and converts prothrombin to thrombin.

The presence of a catalytic surface increases the local concentration of the subcomponents required for the reactions, as well as putting them in juxtaposition. These decrease the Michaelis constant (K_m) of FX and prothrombin, the substrates of tenase and prothrombinase respectively, from far above to far below their respective plasma concentrations. Furthermore, the surface binding may also induce conformation changes in the proteins and help align the substrate cleavage sites with the active site of the enzyme. These enable both reactions to proceed at close to the maximum rate (V_{\max}), which is primarily determined by the co-factors FVIIIa and FVa (Rosing 1980, van Dieijen 1980). The combined decrease in the K_m of the two sequential reactions in the presence of anionic phospholipid surfaces can increase the rate of thrombin formation by several orders of magnitude.

The blood coagulation system is kept in balance by the coagulant and anticoagulant reactions. The binding of thrombin to thrombomodulin on the surface of endothelial cells converts thrombin's procoagulant property to anticoagulant, resulting in the conversion of protein C to activated protein C. Activated protein C then inactivates FVIIIa and FVa, the two co-factors for tenase and prothrombinase complex formation, by limiting proteolysis, inhibiting further generation of thrombin. While protein S is essential for activated protein C to exert its anticoagulant activity in plasma (Esmon 1997), in purified systems the cleavage of FVa and FVIIIa by activated protein C is accelerated less than four-fold (Bakker 1992).

While the ability of MV to act as a procoagulant catalytic surface has been widely studied (Bach 1986, Rosing 1988, Mann 1990, Kalafatis 1994, Andree 1995), reports on their ability to act as an anticoagulant surface are limited. It has been demonstrated that protein S binds to MV but not to the remnant activated platelets, potentiating the binding of activated protein C/protein C to MV. Furthermore, the same protein S binding property was observed in MV generated by different agonists i.e. thrombin, calcium ionophore, and complement C5b-9 (Dahlbäck 1992). Using a purified system, it has been shown that MV also provide a catalytic surface for the inactivation of FVa by activated protein C and protein S in parallel to the formation of prothrombinase complex. In contrast to the first report, both the procoagulant and anticoagulant activities appeared to be dependent on the type of agonists used to generate MV (Tans 1991).

In regard to MV present in platelet concentrates, limited information on their procoagulant property was available prior to the present study. Using platelet factor 3 (PF3) assay, it has been shown that PF3 activity in the supernatant plasma of platelet concentrates increases during storage (Bode 1986). Furthermore, ultracentrifugation of this plasma removed the PF3 activity (Wolf 1967). The PF3 activity, hence, was thought to be associated with the sedimentable material derived from platelets. Nevertheless,

direct detection of MV had not been performed in these reports. The anticoagulant property of MV present in platelet concentrates has never been studied. It is not known whether MV in platelet concentrates generated during processing and storage have the same procoagulant/anticoagulant properties as MV generated by various platelet agonists.

The objective of this study was to investigate whether MV present in platelet concentrates for transfusion contained procoagulant and/or anticoagulant properties, using purified systems for their assessment.

7.2 MATERIALS AND METHODS

7.2.1 Isolation of microvesicles

Ten units of leucocyte depleted platelet concentrates were prepared by Cobe LRS apheresis machine. Samples were taken from each unit on day 1 and day 5. MV were isolated using the following procedure.

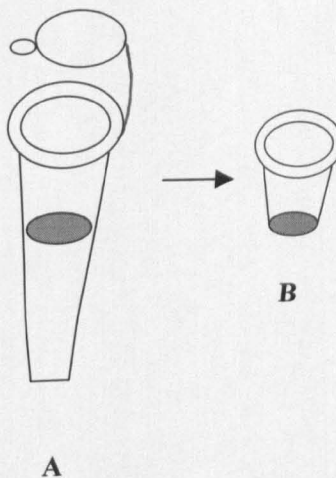
Platelet-free, MV containing plasma was prepared by centrifuging 1 mL of platelet concentrates in a 1.5 polypropylene Eppendorf tube (BDH-Merck, Leicester, UK) at 16100 g (Eppendorf centrifuge 5417C, BDH-Merck, Leicester, UK) for 1 minute. 0.5 mL of the supernatant (MV containing plasma) was saved and MV were measured quantitatively by flow cytometry as described in Chapter 2. Using ultrafiltration, MV were then isolated from the MV-containing plasma by pre-diluting 1 in 6 in 0.05 M Tris/0.1 M NaCl/0.2% BSA (pH 8) to avoid blockage of the filter. The same buffer was also used in all the assays below. 0.4 mL of the diluted MV-containing plasma was filled into a 0.1 μ (0.5 mL holding volume capacity) low protein binding centrifugal filter unit (Millipore S.A., Molsheim, France) (Figure 7.1) and centrifuged at 20300 g for 5 minutes. The filter unit was washed 3 times with Tris/NaCl/BSA and centrifuged at 20300 g for 5 minutes. MV were not detected in the plasma filtrate or wash filtrate,

indicating that negligible amount of MV passed through the filter.

All the assays below were performed in the filter unit containing MV or where stated a blank empty filter.

Figure 7.1 Filter unit used for the isolation of MV.

A centrifugal filter unit consists of a container (B) with a holding volume of 0.5 mL and 0.1 μ filter in the bottom (grey), inside an Eppendorf tube (A). MV were isolated as described in the text above.



7.2.2 Prothrombinase assay

The procoagulant activity of MV was assessed by their ability to act as a catalytic surface for the prothrombinase complex formation. The subcomponents of prothrombinase complex, consisting of FXa, FVa, prothrombin and Ca^{2+} , were added to the test system at a concentration which provided an optimal reaction rate i.e. occurred in a reasonable time as well as being linear in time, while MV provided phospholipids on their membrane and acted as the rate-limiting factor of thrombin generation. The amount of thrombin generated was measured using a chromogenic substrate for thrombin (S2238).

The working concentrations of bovine FXa (Chromogenix AB, Mölndal,

Sweden), and human prothrombin (Enzyme Research Laboratories Ltd, Swansea, UK) were 0.4 and 0.7 U/mL, respectively, in Tris/NaCl/BSA. The working concentration of bovine FVa (Enzyme Research Laboratories Ltd, Swansea, UK) was 1.4 U/mL in Tris/NaCl/BSA/12.5 mM CaCl₂. The substrate S2238 (Chromogenix AB, Mölndal, Sweden) was reconstituted in distilled water to obtain the concentration of 0.50 mM. The final concentrations in the reaction were 0.05 U/mL FXa, 0.2 U/mL FVa, 0.1 U/mL prothrombin, 2 mM CaCl₂, and 0.25 mM S2238.

100 µL Tris/NaCl/BSA pre-warmed at 37°C were added to the filter unit containing MV, followed by 25 µL of each subcomponent, FXa, FVa and prothrombin. The filter unit was gently vortexed before being incubated at 37°C for 10 minutes. The reaction was stopped by the addition of 25 µL Na₂EDTA into the filter unit, followed by gently vortexing. 100 µL of the reaction mixture were transferred to a flat bottom microtitre plate well. 100 µL of substrate S2238 pre-warmed at 37°C were added into each well. The absorbance at 405 nm was recorded every 1 minute for 10 minutes at 22 °C. The initial reaction rate was calculated from the change of absorbance per minute in the first 5 minutes and read against the standard curve plotted from the dilutions of human thrombin (National Institute of Biological Standard and Control, Potters Bar, UK) reconstituted and diluted to 1 U/ml and serially diluted 0.5, 0.25, 0.125, 0.0625 U/mL. Results were expressed as thrombin activity generated.

Serial dilutions of purified phospholipids obtained from human brain (Thrombosis Reference Centre, Manchester, UK) were used to demonstrate the phospholipid dose dependent of the assay. The molar ratio of phospholipid subclasses were 30% phosphatidylcholine, 10% sphingomyelin, 2% phosphatidylinositol, 38% phosphatidylethanolamine, and 20% phosphatidylserine (Stevenson 1986).

Serial dilutions of MV were also used to assess whether the thrombin generated in the assay condition used was MV concentration dependent.

7.2.3 Protein Case assay

The working concentrations of human protein C (Chromogenix AB, Mölndal, Sweden), human protein S (Enzyme Research Laboratories Ltd, Swansea, UK), recombinant human thrombomodulin (Alpha Laboratories Ltd, Hampshire, UK), human thrombin (National Institute of Biological Standard and Control, Potters Bar, UK), and CaCl_2 , were 280 nM, 700 nM, 210 nM, 35 nM, and 2 mM, respectively, in Tris/NaCl/BSA. Substrate for activated protein C (S2366) and thrombin inhibitor (I2581) were reconstituted in distilled water to obtain the working concentrations of 0.50 mM, and 0.14 mM, respectively. The final concentrations of each reagent in the reaction were 40 nM protein C, 100 nM protein S, 30 nM thrombomodulin, 5 nM thrombin, 0.3 mM CaCl_2 , 0.25 mM S2366, and 0.02 mM I2581.

25 μL Tris/NaCl/BSA pre-warmed at 37°C were added to the filter unit containing MV, followed by 25 μL of each subcomponent, protein C, protein S, thrombomodulin, thrombin, and CaCl_2 . The filter unit was gently vortexed before being incubated at 37°C for 10 minutes. 25 μL I2581 was added to inhibit the amidolytic activity of thrombin which is 75% of activated protein C. The filter unit was vortexed gently and incubated for further 3 minutes. 100 μL of the mixture were transferred to a flat bottom microtitre plate well. 100 μL of substrate S2366 pre-warmed at 37°C were added into each well. The absorbance at 405 nm was recorded every 1 minute for 10 minutes. The initial reaction rate was calculated from the change of absorbance per minute in the first 5 minutes and read against the standard curve plotted from the dilutions of human activated protein C (Chromogenix AB, Mölndal, Sweden) serially diluted to 100, 50, 25, 12.5, 6.25 nM. Results were expressed as activated protein C activity generated.

As thrombin has 75% of activated protein C amidolytic activity using substrate S2366, a blank filter unit containing MV and all subcomponents except protein C was also

tested to assess the amidolytic activity of residual thrombin. Furthermore, investigate whether MV were required for the generation of activated protein C, the assay was also performed in a filter without the presence of MV.

7.2.4 Activated protein C inactivation of FVa

The working and final concentrations of FXa, FVa, prothrombin, CaCl_2 , and substrate S2238 were similar to the prothrombinase assay. The working and final concentration of activated protein C was 1.12 nM and 0.16 nM, respectively. 75 μL Tris/NaCl/BSA pre-warmed at 37°C were added to the filter unit containing MV, followed by 25 μL FVa containing CaCl_2 and 25 μL of activated C or Tris/NaCl/BSA. The filter unit was gently vortexed before being incubated at 37°C for 3 minutes. 25 μL of FXa, and prothrombin were added. The filter unit was gently vortexed before being incubated at 37°C for further 10 minutes. 100 μL of the mixture were transferred to a flat bottom microtitre plate well. The thrombin generated was measured using the same procedure as the prothrombinase assay. Results on FVa inhibition were expressed as the percent reduction in thrombin generated in the presence of activated protein C against that in the absence of activated protein C.

All assays were performed in duplicate. If the CV between duplicates was greater than 10%, the results were rejected and the assays were repeated.

7.2.5 Statistical analysis

Wilcoxon matched pairs test was used to compared results between day 1 and day 5. Spearman test was used for the correlation between two set of results.

7.3 RESULTS

7.3.1 Prothrombinase activity

Using purified phospholipids, the phospholipid dose dependent generation of thrombin is shown in Figure 7.2.

A dose-dependent response similar to that seen with purified phospholipids was observed with MV serial dilutions, confirming the optimal conditions of the assay (Figure 7.3).

MV isolated from day 1 platelets showed a significant correlation with the amount of thrombin generated ($r=0.8909$, $p=0.0003$, Figure 7.4). When MV were isolated from the same units of platelets after 5 day storage, all samples showed significantly increased levels of thrombin generated ($p=0.0059$, day 1 vs day 5) as well as MV ($p=0.002$, day 1 vs day 5) (Figure 7.5). However, no correlation in individual samples was seen between MV and the thrombin generated.

7.3.2 Protein Case activity

As the rate-limiting factor for the generation of activated protein C is the binding of protein C to thrombomodulin, not phospholipids, it was not possible to design an assay which showed the phospholipid dose dependent effect. In the conditions used in this study, MV isolated from day 1 and day 5 platelet concentrates generated the same levels of activated protein C, approximately 25-30 nM. (Figure 7.6). No MV concentration dependent effect was seen on day 1 or day 5. Furthermore, a similar amount of activated protein C was generated in a blank filter unit without MV.

7.3.3 Activated protein C inactivation of FVa

MV isolated from day 1 platelets expressed the ability to act as a catalytic surface for FVa inactivation by activated protein C, reducing by 15-30% the level of thrombin generated, as compared to the thrombin generated in the absence of activated protein C. On day 5, the percent reduction in the amount of thrombin generated in the presence of activated protein C was decreased to less than 10%. Furthermore, in some samples the amidolytic activities were slightly higher in the presence of activated protein C due to the cross reactivity of activated protein C with S2238 (Figure 7.7). No correlation was seen between MV and the percent reduction in thrombin generated.

Figure 7.2 The phospholipid dose dependent generation of thrombin.

Serial concentrations of purified phospholipids were used in the prothrombinase reaction.

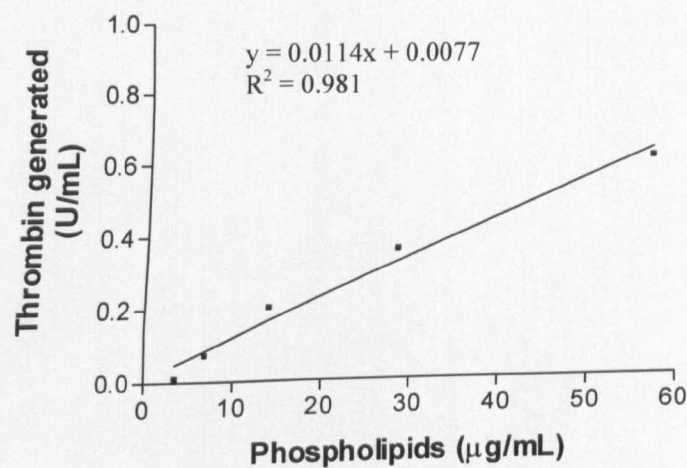


Figure 7.3 The MV concentration dependent generation of thrombin.

Serial dilutions of MV were used in the prothrombinase reaction.

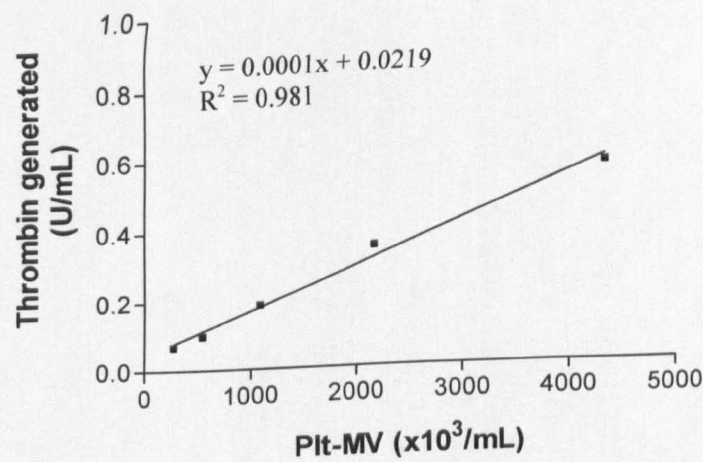


Figure 7.4 Correlation between MV concentration and the amount of thrombin generated on day 1.

$$r=0.8909, p=0.0006$$

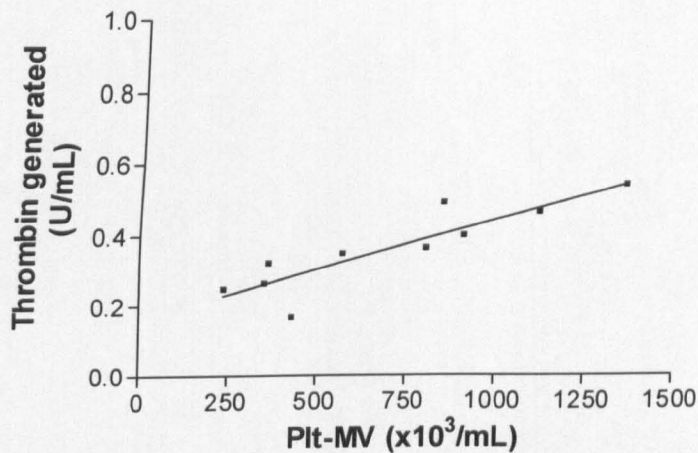


Figure 7.5 MV and thrombin generated on day 1 and day 5.

— Median, Left-Plt-MV, right-thrombin generated. Both MV ($p=0.002$) and the level of thrombin generated ($p=0.0059$) increased on day 5 as compared to day 1.

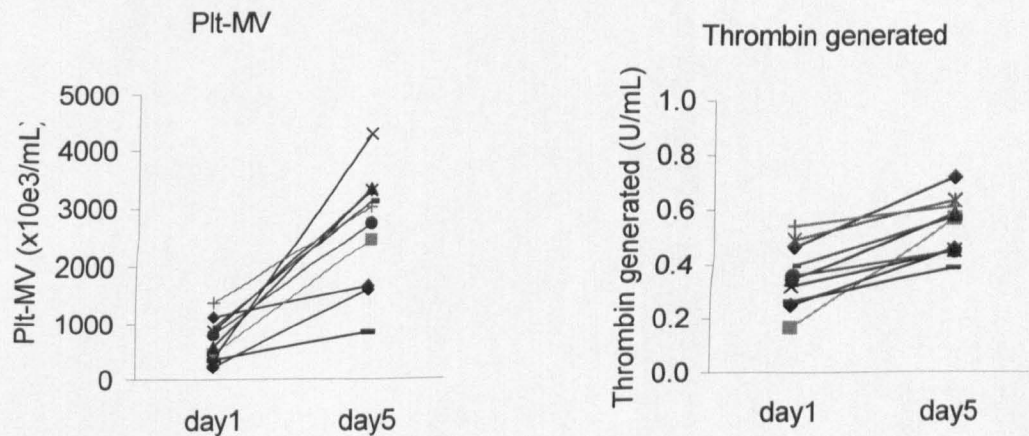


Figure 7.6 Activated protein C generation on day 1 and day 5.
Similar levels of activated protein C were generated on day 1 and day 5

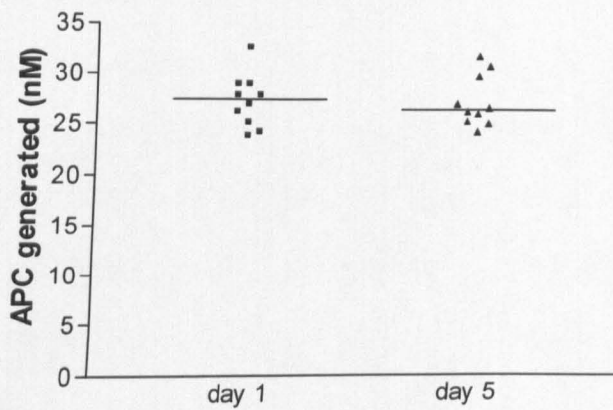
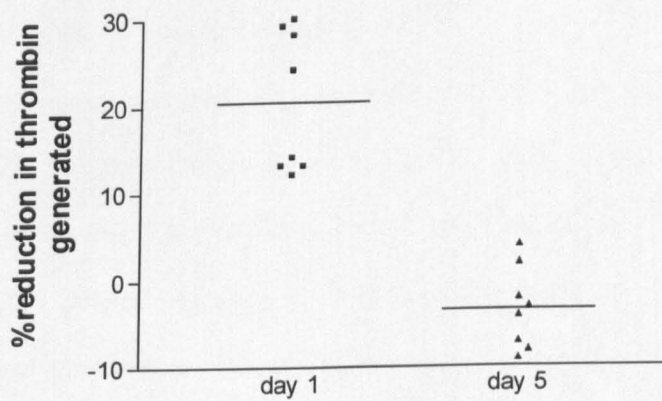


Figure 7.7 Activated protein C inactivation of FVa on day 1 and day 5.
No inactivation of FVa was seen on day 5.



7.4 DISCUSSION

The phospholipids on the membrane of activated platelets as well as MV play essential roles in the coagulant/anticoagulant reactions. In this respect, anionic phospholipids, in particular phosphatidyl serine, contain the highest procoagulant property and act as a rate-limiting factor in the generation of tenase and prothrombinase complex (Mann 1988). Nevertheless, phosphatidylserine also participate in the inactivation of FVa by activated protein C, although the presence of phosphatidylethanolamine is also essential for the anticoagulant property of MV (Smirnov 1999).

Studies have shown that MV generated by various platelet agonists can act as a catalytic surface for both procoagulant and anticoagulant reactions (Tans 1991, Dahlbäck 1992). In this study, MV generated during processing and storage of platelet concentrates also showed the ability to act as a catalytic surface for both reactions. However, only MV isolated from day 1 platelets correlated with the prothrombinase activity. This could possibly be because the phospholipid composition of MV present on day 1 and day 5 was different and that the phosphatidylserine content might be correlated with MV level on day 1 but not on day 5 due to the rearrangement of phospholipids. Furthermore, it could be that MV isolated from day 5 platelets were more heterogeneous in terms of phospholipid distribution. It is not known whether MV present in day 1 and day 5 platelets are generated by the same mechanism. It has been demonstrated that MV generated by different mechanisms, i.e. activation vs non-activation have different characteristic properties. While MV generated by platelet activation express P-selectin on their membranes, MV generated by cellular fragmentation do not (Nomura 1993). In addition, MV formed upon platelet activation contained platelet form of FXIII, whereas MV formed upon mechanical-induced fragmentation contained both platelet and plasma forms (Holme 1993). Furthermore, it has been demonstrated that even MV generated

by the same platelet agonist i.e. thrombin are heterogeneous, some express P-selectin, CD63, and phosphatidylserine on their membranes, other did not (Heijnen 1999). MV present in platelet concentrates were likely to be generated by multiple mechanisms, and hence they may be heterogeneous in terms of their characteristic properties including the phospholipid distribution on their membranes.

With regard to the inactivation of FVa by activated protein C, MV isolated from day 1 platelets showed the ability to act as a catalytic surface for this reaction. However, on day 5, this procoagulant property decreased substantially. This could be due to the inhibitory effects of high phospholipid concentrations and/or high phosphatidylserine molar ratio on the inactivation of FVa by activated protein C (Bakker 1992). The procoagulant activity measured by the reduction in the amount of thrombin generated in the prothrombinase reaction in the presence and absence of activated protein C did not correlate with MV levels. This may be because the phosphatidylethanolamine content, an essential phospholipid for this reaction (Smirnov 1999) was heterogeneous, and did not correlate with the level of MV.

The role of phospholipid membranes in the generation of activated protein C is unclear. Two models of phospholipid participation in activated protein C generation have been proposed: i) protein C interacts directly with phospholipids, leading to an increased affinity of protein C for thrombin-thrombomodulin; and ii) protein C does not directly interact with phospholipids but binds to thrombomodulin that is conformationally changed by interaction with phospholipids (Freyssinet 1986, Galvin 1987, Horie 1994). Furthermore, it has been demonstrated that phosphatidylcholine, a neutral lipid, vesicles inserted with thrombomodulin can act as a surface for the binding of protein C and subsequent activated protein C generation but the binding of protein C to thrombomodulin, not phospholipids, is the rate limiting factor; and that phosphatidylserine is not an essential phospholipid for this reaction (Galvin 1987, Horie

1994). In addition, it has been reported that lipid vesicles containing phosphatidylcholine/phosphatidylethanolamine catalyse protein C activation by thrombin-thrombomodulin with the highest rate as compared to other lipid vesicles (Horie 1994). More recently, the role of anionic phospholipids in the generation of activated protein C in vivo has been challenged. It has been proposed that plasma protein C binds to endothelial cell protein C receptor, which then presents protein C to thrombin-thrombomodulin complex for activation, and that these interactions do not require negatively charged membranes (Sterns-Kurosawa 1996, Esmon 1997).

In the study, it was found that MV isolated from platelet concentrates did not enhance the protein Case reaction as compared to the same assay system performed in the absence of MV. This support the hypothesis that the binding of protein C to thrombomodulin, not phospholipid contents, is the key step and the true rate limiting factor in the generation of activated protein C, and that the generation of activated protein C does not require phospholipid membrane.

In summary, using purified systems this study has demonstrated that MV present in platelet concentrates can act as a catalytic surface for both procoagulant and anticoagulant reactions. While the procoagulant and anticoagulant activities of MV isolated from day 1 platelets appeared to be parallel, the procoagulant activity of MV isolated from the same products after 5-day storage became more pronounced.

CHAPTER 8

THE APPLICATION OF ANNEXIN V AS A NEW MARKER OF PLATELET STORAGE LESION

8.1 INTRODUCTION

Annexin V is a cytosolic glycoprotein found in various cells including blood cells (Reutelingsperger 1988, Kaetzel 1989, Flaherty 1990, Romisch 1992). Although the physiologic function of annexin V is unknown, several studies have demonstrated its anticoagulant property in vitro. Annexin V is a potent inhibitor of both FX and prothrombin binding to activated platelets (Reutelingsperger 1985, van Heerde 1994a, London 1996, Scandura 1996). In addition, it has been shown that in venous blood flow conditions, *annexin V inhibits* platelet adhesion possibly by the reduction of thrombin formation and fibrin deposition (van Heerde 1994b). It has also been shown that *annexin V inhibits* protein kinase C (Schlaepfer 1992) and phospholipase A₂ activities (Haigler 1987). Furthermore, it has been proposed that annexin V may play a role in membrane cytoskeleton function as it is copurified with cytoskeletal proteins (Tzima 1997).

Low levels of annexin V are found in normal citrated plasma (1.1 ± 1.7 ng/mL). Markedly increased levels are found in antiphospholipid syndrome, in particular systemic lupus erythematosus (SLE), in which high levels of anti-annexin V antibody are also found (Kaburaki 1997). Moderately increased levels are found in myocardial infarction (Romisch 1992). The level of annexin V in platelet concentrates has never been studied.

Annexin V is present in different types of blood cells at various concentrations i.e. 2.5×10^{-16} , 2.8×10^{-14} , and 5.9×10^{-16} g/cell for red blood cells, leucocytes, and platelets,

respectively (Romisch 1992). As the numbers of red blood cells and leucocytes in buffy coat derived and apheresis platelet concentrates are much lower than numbers of platelets i.e. red cells $<1 \times 10^9/\text{L}$, leucocytes $<1 \times 10^8/\text{L}$, and platelets $\sim 1 \times 10^{12}/\text{L}$, an increased level of annexin V can be attributed to platelets as the main source. In platelets annexin V is not located in the granules as activation of platelets by strong agonists does not induce the secretion of annexin V (Murphy 1992). It has been proposed that at the intracellular concentration of Ca^{2+} ($0.8 \mu\text{M}$) seen in platelets stimulated by a physiological agonist, such as thrombin 0.1 unit/mL , annexin V is associated with platelet membrane cytoskeleton via annexin V binding protein and is released into the plasma upon membrane damage and/or proteolysis of the membrane (Trotter 1995). During storage of citrate anticoagulated platelet concentrates for transfusion, a similar level of intracellular Ca^{2+} is also observed (Sasakawa 1986), hence changes in the level of annexin V might be useful as a marker of platelet injury/microvesiculation.

The objectives of this study were:

- i. To investigate the level of annexin V in non-leucocyte depleted and leucocyte depleted pooled buffy coat derived platelet concentrates prepared by three leucocyte removal filters.
- ii. To compare the level of annexin V in leucocyte depleted platelet concentrates prepared by Cobe and MCS+ LD apheresis platelets, and Autostop filtered pooled buffy coat derived platelets, the three methods used in routine production.
- iii. To investigate whether annexin V could be used as a surrogate marker of platelet injury/microvesiculation.

8.2 MATERIALS AND METHODS

Ten sets of pooled buffy coat derived platelet concentrates were prepared. Each set consisted of 4 units of platelet concentrates made from the same pool of buffy coats. One unit served as a non-filtered control, and one of the other three was filtered with a negatively charged polyester filter (Autostop) or a positively charged polyester filter (PLX5) or a neutrally charged polyurethane filter (Imugard III). The detailed method was described in Chapter 4. In addition, 10 units of Cobe LRS and MCS+LD (filtered with the LRF6H filter) apheresis platelets were also analysed.

Annexin V was measured in the supernatant plasma of platelet concentrates on day 1 and day 5, using ELISA as described in Chapter 2.

The concentrations of annexin V in leucocyte depleted pooled buffy coat derived platelets prepared by the three different filters as well as unfiltered control were compared using Friedman ANOVA test. The results between leucocyte depleted platelet concentrates prepared by three different technologies i.e. Cobe, MCS, Autostop filtered platelets, were also compared using Kruskal-Wallis ANOVA test. Spearman test was used for the correlation between annexin V and platelet count, microvesicles or soluble P-selectin.

8.3 RESULTS

8.3.1 Comparison between control and three filtered pooled buffy coat derived platelets

On day 1, the levels in control and the three filtered platelets were comparable. By day 5, the levels had increased approximately 2 fold from day 1 values in control, Autostop and PLX5. However, Imugard III filtered platelets gave significantly lower values than control ($p < 0.001$) and Autostop platelets ($p < 0.01$).

8.3.2 Comparison between Cobe, MCS, and Autostop filtered platelets

On day 1, annexin V in Autostop filtered pooled buffy coat derived platelets was higher than Cobe ($p<0.01$) and MCS ($p<0.001$) apheresis platelets. By day 5, both Cobe ($p<0.05$) and Autostop ($p<0.001$) were higher than MCS (Figure 8.2).

8.3.3 Correlation between platelet count, microvesicles, soluble P-selectin and annexin V

No correlation was found between platelet count and annexin V in platelets prepared by buffy coat or apheresis method. However, there was a significant correlation between the values of Plt-MV and annexin V in platelets prepared by buffy coat method ($r=0.7328$, $p<0.0001$, control and three filtered platelets day 1 and day 5 pooled) (Figure 8.3). A significant correlation between Plt-MV and annexin V was also seen in apheresis platelets ($r=0.6233$, $p<0.0001$, Cobe and MCS day 1 and day 5 pooled). (Figures 8.4) No correlation between RBC-MV and annexin V was found.

Soluble P-selectin was correlated with annexin V in both platelets prepared by buffy coat ($r=0.7782$, $p<0.0001$, all data from day 1 and day 5 pooled) and apheresis method ($r=0.6233$, $p<0.0001$, Cobe and MCS day 1 and day 5 pooled). (Figures 8.5, 8.6)

Figure 8.1 Annexin V in control and three filtered buffy coat derived platelets
on day 1 and day 5.

_____ Median. Top-day 1, bottom-day 5. No difference was seen on day 1. On day 5 Imugard III was lower than control ($p<0.001$) and Autostop platelets ($p<0.01$).

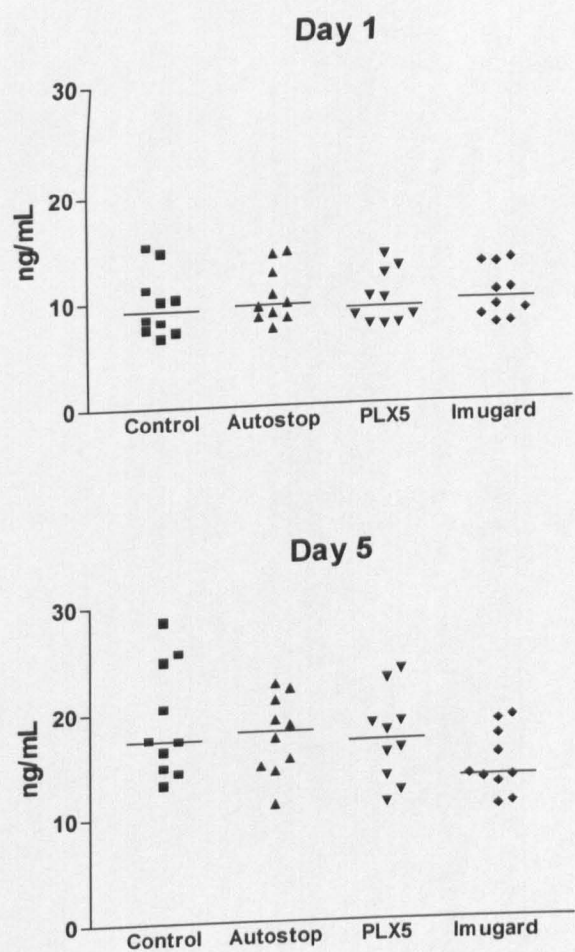


Figure 8.2 **Annexin V in Cobe, MCS and Autostop filtered platelets on day 1 and day 5.**

— Median, top-day 1, bottom-day 5. Day 1, Autostop was higher than Cobe ($p<0.01$) and MCS ($p<0.001$). Day 5, MCS was lower than Cobe ($p<0.05$) and Autostop ($p<0.001$).

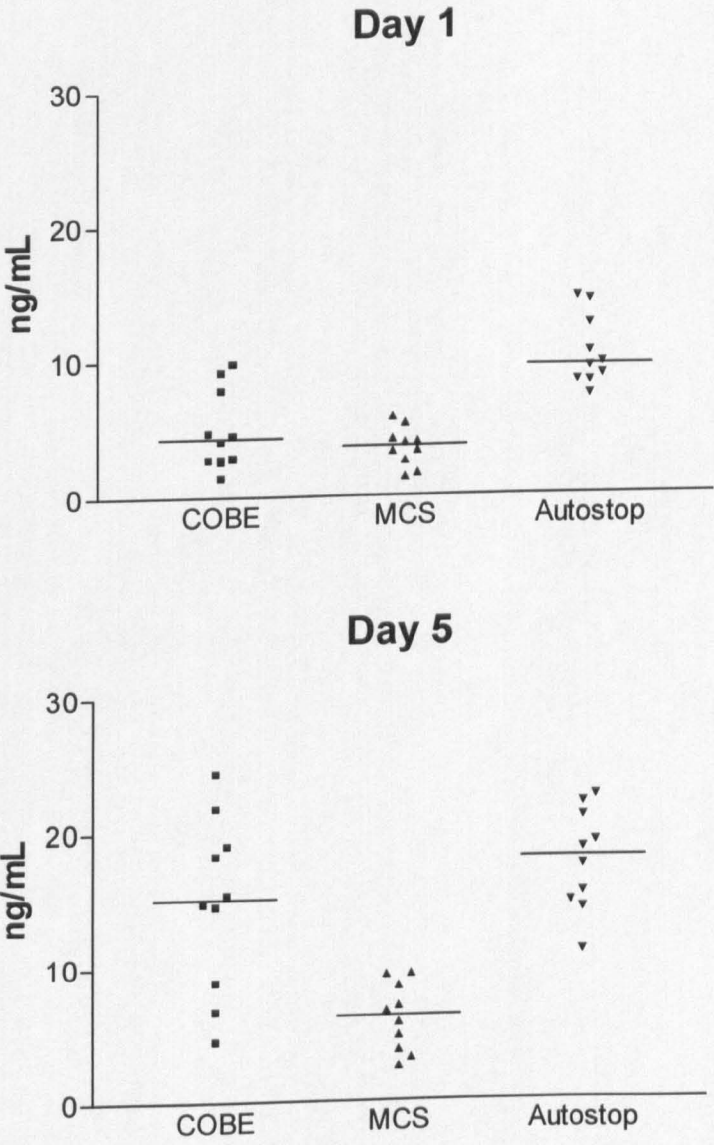


Figure 8.3 Correlation between Plt-MV and annexin V in control and filtered platelets prepared by buffy coat method.

$r=0.7328, p<0.0001$, all data day 1 and day 5 pooled.

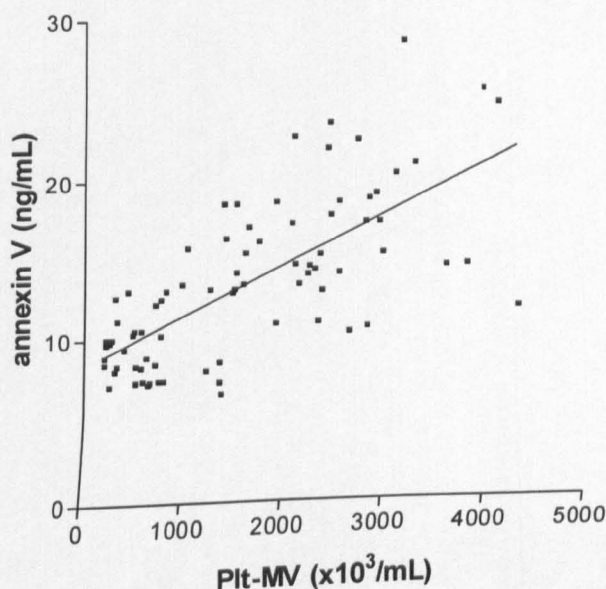


Figure 8.4 Correlation between Plt-MV and annexin V in apheresis platelets.

$r=0.6233, p<0.0001$, Cobe and MCS day 1 and day 5 pooled.

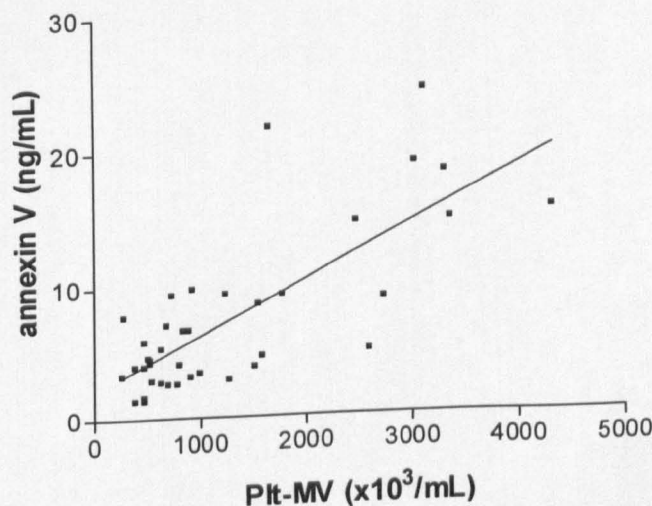


Figure 8.5 Correlation between soluble P-selectin and annexin V in control and filtered platelets.

$r=0.7792, p<0.0001$, all data day1 and day 5 pooled.

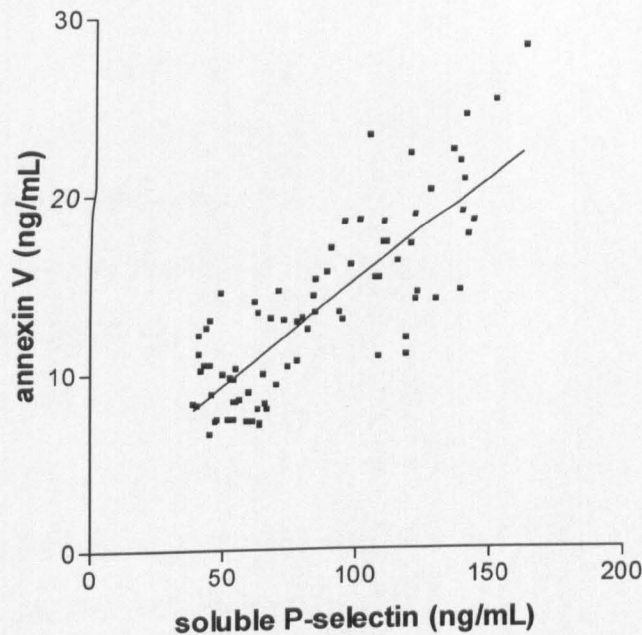
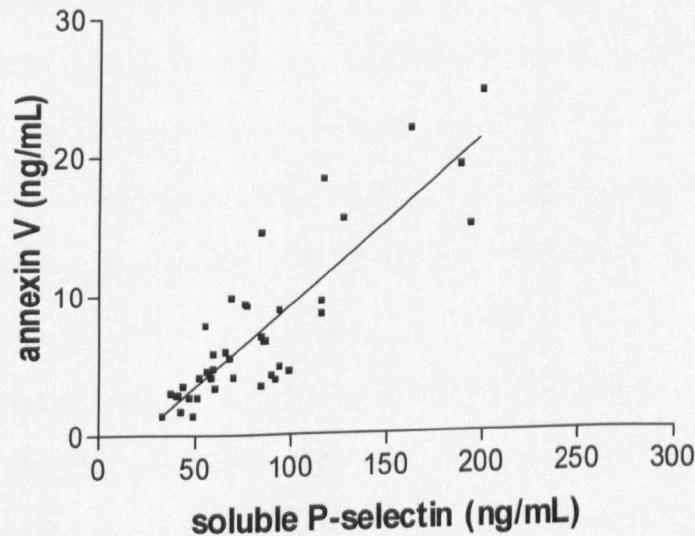


Figure 8.6 Correlation between soluble P-selectin and annexin V in apheresis platelets.

$r=0.8053, p<0.0001$, Cobe and MCS day 1 and day 5 pooled.



8.3 DISCUSSION

Upon activation, platelets undergo changes in internal reorganisation, secretion of granule contents and aggregation. Activation by platelet agonists, such as thrombin, stimulates signal transduction pathways, which result in an increase in cytosolic Ca^{2+} . However, the molecular events, which lead to the increase in Ca^{2+} , remain unknown. In platelet concentrates, during storage platelet activation caused by several factors, such as the interaction with serine proteases, also leads to the increase in cytoplasmic Ca^{2+} (Sasakawa 1986, Bode 1989).

In resting platelets, annexin V is thought to be located in the cytoplasm. In the presence of $0.8 \mu\text{M}$ of cytoplasmic Ca^{2+} , the level seen in platelets stimulated by physiological agonists as well as in stored platelet concentrates, annexin V binds to the membrane cytoskeleton via a 50kDa annexin V binding protein (Trotter 1995). Concomitantly, the increased cytoplasmic Ca^{2+} can also lead to the activation of calpain, a calcium dependent enzyme, resulting in the proteolysis of membrane cytoskeleton, the release of annexin V from the membrane, and/or microvesiculation.

The levels of annexin V in the supernatant plasma of platelet concentrates on day 1 were approximately 2-5 times and further increased to 5-10 times higher than normal plasma levels during the 5-day storage. Both control and the three filtered pooled buffy coat derived platelets did not show any difference in annexin V on the day of production (day 1) implying that either filtration did not cause membrane damage which could result in the increased level of annexin V, or filtration both generated and removed annexin V resulting in no net effect.

The higher level of annexin V seen in Autostop filtered pooled buffy coat derived platelets as compared to both Cobe and MCS apheresis PC on day 1 was possibly because during the preparation of platelets by buffy coat method, buffy coats containing

high concentrations of red blood cells, leucocytes and platelets were kept overnight at 22°C. The enzymes released from leucocytes such as elastase and cathepsin could cause proteolysis of red cell and platelet membranes, leading to the release of annexin V. However, the lower level of leucocytes present in the control unfiltered platelets (less than 100 leucocytes/ μ L) did not influence annexin V levels on storage as no difference was seen between control and Autostop filtered platelets, both were kept in the same type of storage bag. This implied that the level of leucocytes present in unfiltered platelets prepared by pooled buffy coat method did not influence platelet storage lesion. Furthermore, it indicated that platelets were the main source of the increase in annexin V. This was further substantiated by the finding that the higher level of RBC-MV in control as compared to Autostop filtered platelets on storage did not lead to the higher level of annexin V. In addition, considering the numbers of leucocytes and red cells present in platelet concentrates, even with the complete cell lysis and total release of annexin V from these cells, it would only amount to less than 3 ng/mL of annexin V.

A similar trend to annexin V was also observed with Plt-MV and P-selectin in control and Autostop filtered platelets on storage. Furthermore, Imugard filtered platelets, which showed the lowest level of annexin V on storage, also showed the lowest levels of Plt-MV and P-selectin, as compared to other groups of pooled buffy coat derived platelets. Cobe and Autostop platelets gave higher values of annexin V, Plt-MV and P-selectin than MCS.

Although the release of annexin V is not directly platelet activation dependent i.e. no secretory-fusion occurs, the correlation with soluble P-selectin, an activation dependent marker, implies that activation has an indirect role by causing the proteolysis of membrane and/or microvesiculation. Furthermore, the correlation of annexin V with Plt-MV levels between the two parameters indicated that they shared some common mechanisms of generation

Several anticoagulant properties of annexin V have been demonstrated *in vitro*, but its physiologic role remains unknown. In the presence of 5 mM of CaCl_2 annexin V has a high affinity binding to phosphatidyl serine on the membrane of activated platelets, inhibiting the binding of FX and prothrombin. The mechanism of the inhibition has not been clarified but it is not by a simple competition (Scandura 1996). Antibodies to annexin V have been found in a number of patients with antiphospholipid syndrome (de Groot 1993, Matsuda 1994, Kaburaki 1997). It has been hypothesised that anti-annexin V antibodies inhibits its anticoagulant property (Kaburaki 1997, Rand 1998). This might partly play a role in the thrombotic tendency seen in these patients.

The clinical significance of the annexin V present in platelet concentrates is unknown. It is likely that after transfusion it will bind to anionic phospholipid membrane of activated platelets or MV due to the high concentration of Ca^{2+} , i.e. millimolar levels, in the circulation. Although under an optimal storage condition the amount of annexin V released was less than 10% of total annexin V content in platelets, suboptimal storage conditions may lead to higher levels of platelet activation/microvesiculation, and hence higher levels of annexin V. Transfusion of such platelets may reduce the haemostatic effectiveness of the products.

As the changes in annexin V levels in platelet concentrates are directly and indirectly related to platelet activation/microvesiculation/fragmentation, it may be useful as a marker to detect the overall platelet storage lesion.

Since November 1999 all blood components in the UK have been subjected to leucocyte depletion, as a precaution against the theoretical possibility of variant Creutzfeldt-Jacob disease (vCJD) transmission. Leucocyte depleted platelet concentrates are currently prepared by three major methods, i.e. leucocyte filtration of pooled buffy coat derived platelets, leucocyte filtration of Haemonetics MCS+ LD apheresis platelets, and Cobe LRS apheresis platelets. Leucocyte depletion in the last method is achieved without the use of leucocyte removal filter, but employs the fluidised particle bed principle.

Using an automated blood cell counter and a simple procedure, in which citrate anticoagulated platelets were exposed to EDTA, and platelet size distribution indices of citrate and citrate plus EDTA samples were compared, it has been demonstrated platelets prepared by various methods mentioned above responded differently to EDTA. This implied that platelets recovered from different preparation methods contained different levels of functional reserve. In this respect, platelets prepared by buffy coat method appeared to be the most reactive, whereas Cobe apheresis platelets were the least.

Isolation of platelets for transfusion, from whole blood by any methods resulted in some degree of platelet activation and microvesiculation, which were further increased during storage. This study has quantitated platelet-derive microvesicles in platelet concentrates for transfusion for the first time. Although prior to this study, there have been reports on platelet-derived microvesicles in platelet concentrates, the measurements were carried out qualitatively (Wolf 1967, Solberg 1987, Bode 1991, 1994).

The effects of leucocyte filtration on platelet activation and microvesiculation

have never been explored. Leucocyte removal filters made from three different synthetic materials, namely negatively charged polyester (Autostop), positively charged polyester (PLX5), and neutrally charged polyurethane (Imugard III), were investigated. An originally designed method for the preparation of four groups of platelet concentrates derived from the same pool of buffy coats was used to enable the comparison of three in-process leucocyte removal filter/storage bag combinations currently available in the market, as well as unfiltered control. None of the three leucocyte removal filters used gave a net effect in terms of platelet activation or platelet-derived microvesicles generation/removal. During storage, the levels of platelet activation and microvesiculation increased in all types of platelets. The increase in platelet activation and microvesiculation were remarkably influenced by the volume and surface area of the storage bags used. Storage bag with the lowest volume and surface area showed the lowest levels of platelet activation/microvesiculation. This was substantiated by the finding that the difference among the three filtered and unfiltered platelets were eliminated when all four types of platelets were stored in the same type of bag. Furthermore, the levels of residual leucocyte seen in the unfiltered platelets or filtration did not influence the degree of platelet activation/microvesiculation during storage if both unfiltered and filtered products were kept in the same condition.

Platelets prepared by different methods did not show any difference in terms of platelet activation/microvesiculation at the beginning of shelf-life. However, at the end of the 5-day storage, platelets prepared by Haemonetics MCS+ LD apheresis technique showed the lowest level of platelet activation and platelet-derived microvesicles.

The effect of platelet activation, assessed by the expression of P-selectin on platelet membrane, on the survival of transfused platelets is equivocal. Earlier reports suggested a shortened survival of activated platelets expressing P-selectin (Rinder 1991, Triulzi 1992, Holme 1997). The mechanism involved is thought to be the binding of

activated platelets to leucocytes and their subsequent clearance from the circulation either by the mononuclear-phagocyte system or by entrapment of the platelet-leucocyte aggregates in the microcirculation. Nevertheless, recent reports have shown that P-selectin deficient mice clear activated platelets at the same rate as normal mice (Berger 1998) and that P-selectin positive platelets have normal survival in baboons (Michelson 1996). It has also been shown that after transfusion, P-selectin on the platelet membrane is rapidly cleaved into the plasma to become the soluble form (Berger 1998). These suggest that P-selectin expression per se may not directly account for in vivo survival of transfused platelets. Nevertheless, the expression of P-selectin on platelet membrane may have some biological implication as it has been shown to be associated with the inflammatory process by recruiting monocytes and neutrophils to the site of tissue injury (Larsen 1989, Hamburger 1990). More recently, it has been reported that platelet-derived microvesicles expressing P-selectin mediate leucocyte-leucocyte interactions on endothelium (Forlow 2000).

The physiologic role of plasma soluble P-selectin is not clear. It is believed that it may reduce complement-mediated ischemia-reperfusion injury (Woodcock 1998). The P-selectin molecule contains nine short consensus repeats, which are common to proteins that bind the C3b and iC3b components of the classical pathway of complement (Johnston 1989). By competing with complement activating surfaces, soluble P-selectin may act to reduce complement-mediated injury. It remains to be elucidated if transfusions of platelets expressing surface P-selectin and/or containing plasma soluble P-selectin would have any effects on inflammatory process. Furthermore, it has been proposed that soluble P-selectin could act as an antiadhesive molecule in vivo, by competing with the membrane-bound P-selectin for the same receptor (Stohlawetz 1998). This may have clinical consequences in terms of antithrombotic effect because treatment with a blocking monoclonal antibody to P-selectin has been shown to accelerate

thrombolysis in a primate model of arterial thrombosis (Toombs 1995).

The physiologic significance of platelet derived microvesicles is not clear. Nevertheless, the increased levels of circulating platelet-derived microvesicles have been observed in patients with primary thrombotic disorders such as transient ischemic attacks and myocardial infarction (Lee 1993, Jy 1995), as well as patients with secondary activated coagulation i.e. disseminated intravascular coagulation (DIC) (Holme 1994). Furthermore, high levels of platelet-derived microvesicles have also been reported in thrombotic episodes associated with several other pathological conditions. These include sickle cell disease (Wun 1998), paroxysmal nocturnal haemoglobinuria (Wiedmer 1993, Hugel 1999), heparin induced thrombocytopenia (Warkentin 1994), and idiopathic thrombocytopenic purpura (Jy 1992). The prothrombotic property of platelet-derived microvesicles is attributed to the high proportion of phosphatidylserine on their membrane which enhances the tenase and prothrombinase reactions (Bever 1991). Although studies have shown that platelet-derived microvesicles generated by various platelet agonists contain anticoagulant as well as procoagulant properties (Tan 1991, Dahlbäck 1992), the clinical significance of their anticoagulant property is yet to be demonstrated. Nevertheless, in this study it has been demonstrated for the first time that similarly to microvesicles generated by platelet agonists, microvesicles isolated from relative fresh platelet concentrates i.e. day 1 also contain both procoagulant and anticoagulant properties, but upon 5-day storage the procoagulant activity appears to be more pronounced.

So far only one study has measured quantitatively the level of platelet-derived microvesicles in samples obtained from patients with various pathological conditions (Combes 1997). However, without interlaboratory standardisation, it is not relevant to compare the levels of microvesicles seen in platelet concentrates with those reported in clinical conditions due to differences in several variables employed by both settings

which could influence ex vivo platelet activation/microvesiculation. These include: i) the containers used for sample collection, while clinical samples are collected in a plastic test tube, whole blood for platelet concentrate preparation is collected in a much larger plastic bag, both have different biocompatibility; ii) the type and ratio of anticoagulant; iii) the shear force generated by centrifugation to isolate platelets; and iv) the method used to set the arbitrary region for microvesicles.

More recently, attention has been placed on the finding that platelets are the major source of normal prion protein (PrP^c) (MacGregor 1999, Prowse 1999) and upon activation by platelet agonists, PrP^c in platelets is expressed on the platelet surface in parallel to the platelet activation markers, P-selectin and CD63 (Holada 1999, Perini 1996). Furthermore, ruptured platelets containing P-selectin also contain PrP^c. As abnormal prion protein is known to be membrane associated (Cashman 1990, Bendheim 1992), it remains to be elucidated whether platelet-derived microvesicles could act as a carrier of abnormal prion protein.

Artificial surfaces are known to activate coagulation and complement systems. The effects of leucocyte depletion on the activation of the coagulation system varied according to the assays used, i.e. while prothrombin fragment 1+2 and thrombin-antithrombin complex measured by ELISA did not show any difference between leucocyte depleted platelets prepared by different leucocyte removal filters or by various leucocyte depletion technologies, kallikrein-like and thrombin-like activities measured by chromogenic assays were different. The latter assays are more sensitive and provide the overall information on the activation of coagulation system. The activation of contact system by negatively charged filters can cause hypotensive reactions in transfused patients also receiving angiotensin converting enzyme inhibitors (Fried 1996, Hume 1996, Sano 1996, Mair 1998). The clinical significance of activated complements in platelet concentrates has not been reported. Nevertheless, as some activated complement

components such as C3a have pro-inflammatory effects, they may cause transfusion reactions.

In summary, all types of leucocyte removal filters and leucocyte depletion methods met the specification for leucocyte depletion. None of the leucocyte filters generated or removed platelet-derived microvesicles. Leucocyte depleted platelets prepared by different methods contained similar levels of platelet-derived microvesicles. The negatively charged polyester filter generated low grade serine proteases but removed activated complement component C3a. The positively charged polyester filter generated C3a. The neutrally charged filter did not affect the levels of serine proteases or activated complement components.

On one hand, it is plausible to conclude that an ideal product should contain platelets in their native state with the least degree of activation/release reaction and microvesiculation as well as without plasma activation so that platelets can provide optimal haemostatic functions at the site of injury in vivo while minimising transfusion reactions. On the other hand, high levels of activated platelets and microvesicles might be desirable for patients who require prompt haemostatic effectiveness.

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